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13. ABSTRACT (Maximum 200 Words) The Perioperative Neuroprotection Symposium was held on April 16-19 in Ft. Lauderdale, Fl. The meeting was attended by approximately 140 registrants. Twenty four invited speakers made presentations in five sessions. An additional 40 poster presentations were available for discussion throughout the meeting. Each participant received a symposium abstract book that included mini-reviews from each of the speakers and the short abstracts from the posters. The speakers also submitted formal manuscripts for peer-reviewed publication. Five of the poster presenters were also invited to submit short research manuscripts. These articles were published relatively rapidly in the August 2004 issue of the Journal of Bioenergetics and Biomembranes (Volume 36, No. 4). Feedback from the symposium participants was very positive and another similar symposium is planned for the Spring of 2006.				
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Mitochondria and Neuroprotection Symposium

**April 16 – 19, 2004
Ft. Lauderdale Florida
Marriott Harbor Beach Resort & Spa**



In Memory of Albert L. Lehninger

**Organized by
University of Maryland School of Medicine
Department of Anesthesiology and
Program in Neuroscience Neuroprotection Focus Group**



Mitochondria and Neuroprotection Symposium

**April 16 – 19
Ft. Lauderdale Florida
Marriott Harbor Beach Resort & Spa**

Organizers

**University of Maryland
School of Medicine Department of Anesthesiology and
Program in Neuroscience Neuroprotection Focus Group**

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Publication

**Speaker's mini-reviews and 4 research communications
based on select poster presentations to be published in
J. Bioenergetics and Biomembranes late in 2004**

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Mitochondria and Neuroprotection Symposium
April 16-19, 2004





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Albert Lester Lehninger
February 17, 1917 – March 4, 1986

Albert Lehninger was born in Bridgeport, Connecticut and received a B.A. in English from Wesleyan University in 1939. He received his Ph.D. in Physiological Chemistry from the University of Wisconsin in 1942. Lehninger stayed on at the Univ. of Wisconsin as an Instructor until 1945, when he was appointed Assistant Professor of Biochemistry and Surgery at the University of Chicago. At this juncture he had published 11 research articles. From 1945 – 1952, he published an additional 33 articles. These studies included the discovery that fatty acid oxidation, ketone body metabolism, and the TCA cycle occur in the mitochondrion, some of the first investigations employing isolation of this organelle (see e.g., JBC 1949 and 1950). Thus, while Lehninger was a chemist at heart, he was one of the founding fathers of cell biology. In 1952 at the tender age of 33, Albert Lehninger was appointed the Chair of Physiological Chemistry at the Johns Hopkins University School of Medicine and retained that position until 1978. He developed this department into what was and still is one of the most outstanding departments of biochemistry in the world. In the 10 year span of 1952 to 1962, he published 88 articles. These studies helped elucidate the roles of different electron transport chain complexes in energy metabolism and identified the mitochondrion as a site of action of thyroid hormone. Moreover, his research established many principles of metabolic regulation that apply to all aspects of cellular homeostasis. In the next 10 years, he published 100 articles. This era included elucidation of how mitochondrial calcium uptake was coupled to respiration. He and his coworkers also contributed to the understanding of respiratory uncoupling and mitochondrial transport of ATP and ADP during this period. In 1970, he published an article on "Comparative studies on mitochondria isolated from neuron-enriched and glia-enriched fractions of the brain", a topic that is still very timely and closely related to the subject of this symposium. During the next several years, the Lehninger lab expanded on the comparative biology theme, characterizing mitochondrial heterogeneity among different normal tissues, and between normal and neoplastic cells. The 70's was a period of intense controversy concerning the mechanism of oxidative phosphorylation. While Lehninger's research strongly supported Peter Mitchell's chemiosmotic coupling hypothesis, his meticulous measurements of the stoichiometric relationships between oxygen consumption, proton efflux, and calcium and phosphate uptake helped refine the hypothesis into a generally accepted mechanism. The last approximately 10 years of his career focused on the roles of mitochondrial enzymes and transporters in physiological and pathological energy metabolism and cell calcium homeostasis. These investigations included a comparison of the calcium uptake affinities of mitochondria and endoplasmic reticulum, and how mitochondrial respiration is inhibited by a factor released by macrophages, later identified as nitric oxide. In total, Lehninger published 310 research articles. In addition, he was the sole author of 7 books, including "The Mitochondrion" in 1964, the first edition of "Bioenergetics" in 1965, and three editions of his world-famous Biochemistry textbook, published in over 12 different languages. His numerous additional honors and activities include 6 honorary doctorates, election to the National Academy of Sciences, and election as President of the American Society of Biological Chemists. Most importantly, Lehninger trained scores of scientists and educators from every corner of the globe, and was admired and loved by all who had the good fortune to know him. I am proud to be one of these individuals.

Gary Fiskum
Upper Falls, Maryland
April 8, 2004

Mitochondria and Neuroprotection Symposium
April 16-19, 2004





SCIENTIFIC PROGRAM

Friday, April 16

- 5:00 – 7:00 Registration
6:00 – 7:30 Welcome Reception

Saturday, April 17

- 8:00 – 8:45 Continental Breakfast / Registration
8:45 Gary Fiskum – Univ. of Maryland
 Welcome and tribute to Dr. Albert L. Lehninger

9:00 – 12:15 Mitochondrial Biology of Neural Cell Death I
 Chair: Laura Dugan – Washington Univ.

- 9:00 David Nicholls – Buck Institute
 What happens to mitochondria in glutamate-exposed neurons?
9:45 Ian Reynolds – Univ. Pittsburgh
 Mitochondrial trafficking in neurons: a key variable in neurodegeneration?
10:30 Coffee Break
10:45 Valina Dawson – Johns Hopkins Univ.
 Deadly conversations in mitochondrial-nuclear pillow-talk
11:30 Jochen Prehn – Royal College of Surgeons in Ireland
 BH3 only proteins activate mitochondrial cell death pathways during the accumulation of non-functional proteins
12:15 – 1:30 Lunch

1:30 – 6:00 Mitochondrial Biology of Neural Cell Death II
 Chair: David Nicholls – Buck Institute

- 1:30 Janet Dubinsky – Univ. Minnesota
 Changes in mitochondrial Ca²⁺ sensitivity in mutant huntingtin mice
2:15 Michael Norenberg – Univ. Miami
 Ammonia neurotoxicity and the mitochondrial permeability transition
3:00 Bruce Kristal – Burke Institute
 The mitochondrial permeability transition as a target for neuroprotection
3:45 Coffee Break
4:00 Rona Giffard – Stanford Univ.
 Effect of overexpression of protective genes on mitochondrial function of stressed astrocytes
4:45 Laura Dugan – Washington Univ.
 Astrocyte mitochondria in in vitro and in vivo models of ischemia
5:30 Guy Seabrook – Merck & Co.
 Mitochondria and Neuroprotective Drug Development
6:00 – 7:00 Posters and cash bar



Sunday, April 18

8:00 – 8:30 Continental breakfast

8:30 – 12:30

Acute Brain Injury I
Chair: Jim Bennett – Univ. Virginia

- 8:30 Miguel Perez-Pinzon – Univ. of Miami**
Neuroprotective effects of Ischemic preconditioning in brain mitochondria following cerebral ischemia
- 9:15 Neil Sims – Flinders Univ., Australia**
Mitochondrial glutathione: a modulator of brain cell death
- 10:00 Coffee break**
- 10:15 Gary Gibson – Burke Institute**
Mitochondrial enzymes and endoplasmic reticulum calcium as targets of oxidative stress and of therapeutic approaches
- 11:00 Cecilia Giulivi – Univ. Minnesota**
The Janus role of mitochondrial nitric-oxide synthase
- 11:45 Gary Fiskum – Univ. of Maryland**
Oxidative mitochondrial stress impairs post-ischemic cerebral energy metabolism and exacerbates delayed neuronal cell death

12:30 – 3:00 Free time!

3:00 - 6:00

Acute Brain Injury II
Chair: Gary Gibson – Burke Institute

- 3:00 Patrick Sullivan – Univ. Kentucky**
Mitochondrial uncoupling as a therapeutic target following neuronal injury
- 3:45 Liz Jonas – Yale Univ.**
The Hypoxia Channel: Mitochondrial response to acute neuronal injury
- 4:30 Courtney Robertson – Univ. of Maryland**
Mitochondrial dysfunction contributes to cell death following traumatic brain injury in adult and immature animals
- 5:15 Henrik Hagberg – Institute for the Health of Women and Children, Sweden**
Mitochondrial impairment in the developing brain after hypoxia-ischemia

6:30 – 7:30 Posters and cash bar

7:30 – 9:30 Dinner banquet



Monday, April 19

7:30 – 8:00 Continental Breakfast

8:00 – 12:00

Neurodegenerative Diseases

Chair: Valina Dawson – Johns Hopkins Univ.

8:00 Serge Przedborski – Columbia Univ.

MPTP as a mitochondrial neurotoxic model of parkinson's disease

8:45 M. Flint Beal – Cornell

Mitochondria and neurodegenerative diseases: Pathogenesis and treatment

9:30 Tim Greenamyre – Emory Univ.

Complex I and Parkinson's disease

10:15 Coffee Break

10:30 Jim Bennett – Univ. Virginia

Development of mitochondrial gene replacement therapy

11:15 Zuoshang Xu – Univ. Massachusetts

Mitochondrial vacuolation by intermembrane space expansion--a new form of mitochondrial degeneration caused by ALS-associated mutant SOD1

12:00

Meeting adjourns

Mitochondria and Neuroprotection Symposium
April 16-19, 2004





The Integration of Mitochondrial Calcium Transport and Storage

David G. Nicholls and Susan Chalmers

Buck Institute for Age Research, Novato, California, USA

The extraordinary capacity of isolated mitochondria to accumulate Ca^{2+} has been established for more than 40 years. The distinct kinetics of the independent uptake and efflux pathways account for the dual functionality of the transport process to either modulate matrix free Ca^{2+} concentrations or to act as temporary stores of large amounts of Ca^{2+} in the presence of phosphate. One puzzle has been the nature of the matrix calcium phosphate complex, since matrix free Ca^{2+} seems to be buffered in the region of 1-5 μM in the presence of phosphate while millimolar Ca^{2+} remains soluble in *in vitro* media. The key seems to be the elevated matrix pH and the third-power relationship of the PO_4^{3-} concentration with pH. Taking this into account we may now finally have a model that explains the major features of physiological mitochondrial Ca^{2+} transport.

As so ably documented by the studies of Al Lehninger and his collaborators in the 1960's and 1970's, one of the most fascinating properties of isolated mitochondria is their seemingly enormous capacity (frequently in excess of 1 micromole/mg protein - corresponding to a total matrix concentration of 1M) to accumulate and retain calcium. In their classic studies (reviewed in Rossi & Lehninger 1964; Lehninger *et al.* 1967; Lehninger 1974) some of the major features were established, including the role of phosphate as accompanying ion, effects on respiration and the detection of associated proton movements, although this last was initially ascribed to the passive response to a primary Ca^{2+} transport process rather than a manifestation of the chemiosmotic hypothesis. With the gradual realization that this latter provided a mechanism for the uptake of Ca^{2+} via a uniport channel came a new problem, namely that estimates of the membrane potential $\Delta\Psi$ in the region of 150mV (Nicholls 1974) and thus capable of maintaining a 10^5 gradient of free Ca^{2+} across the inner membrane led to the conclusion that a uniport would lead to an irreversible accumulation of the cation into the matrix. This paradox was resolved by the discovery of independent efflux pathways in heart and liver mitochondria (Crompton & Heid 1978; Crompton *et al.* 1978) leading to the concept of a continuous cycling of Ca^{2+} across the membrane utilizing the proton gradient either directly in liver mitochondria, via the $\text{H}^+/\text{Ca}^{2+}$ exchanger, or indirectly in the case of heart or brain mitochondria where a combination of $\text{Na}^+/\text{Ca}^{2+}$ and H^+/Na^+ exchangers were operative (Fig. 1).

This cycling of Ca^{2+} led to a wealth of papers in which the ability of the pathways to transmit changes in cytoplasmic free Ca^{2+} , $[\text{Ca}^{2+}]_c$, into the matrix to control key metabolic enzymes including pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and NAD-linked isocitrate dehydrogenase (reviewed in Monje *et al.* 2001). However it was still important to establish why mitochondria accumulated Ca^{2+} rather than simply cycling it. Our own investigations began with the acquisition of a Ca^{2+} -selective macro-electrode and an investigation of the extent to which mitochondria could reduce the extra-mitochondrial free Ca^{2+} concentration, $[\text{Ca}^{2+}]_e$ (Nicholls 1978). Respiring liver mitochondria incubated in the presence of 30nmol total Ca^{2+} /mg protein could lower $[\text{Ca}^{2+}]_e$ to about 0.8 μM while if $[\text{Ca}^{2+}]_e$ was lowered below this value by the addition of a chelator there was a slow release of matrix Ca^{2+} until this same value was attained (Nicholls 1978). It thus seemed that this represented a value at which a dynamic steady-state equilibrium cycling was achieved.



The equilibrium $[Ca^{2+}]_e$, which we termed the 'set-point' was invariant when the total Ca^{2+} load was varied from 10 to 50nmol/mg protein (Nicholls 1978). However above this value the capacity of the mitochondria to accumulate Ca^{2+} was severely curtailed and the set-point rose. Net accumulation of Ca^{2+} would be predicted to lower $\Delta\Psi$ and raise ΔpH as net proton extrusion occurs in compensation for the uptake of Ca^{2+} . The only reason why 50nmol/mg protein of Ca^{2+} could be accumulated without affecting the set-point was because of the presence of endogenous phosphate in the preparation. When this was depleted by preincubating mitochondria with glucose and hexokinase or by inhibiting the phosphate transporter with N-ethylmaleimide much less Ca^{2+} could be taken up before the set-point was raised (Nicholls 1978; Zoccarato and Nicholls 1982).

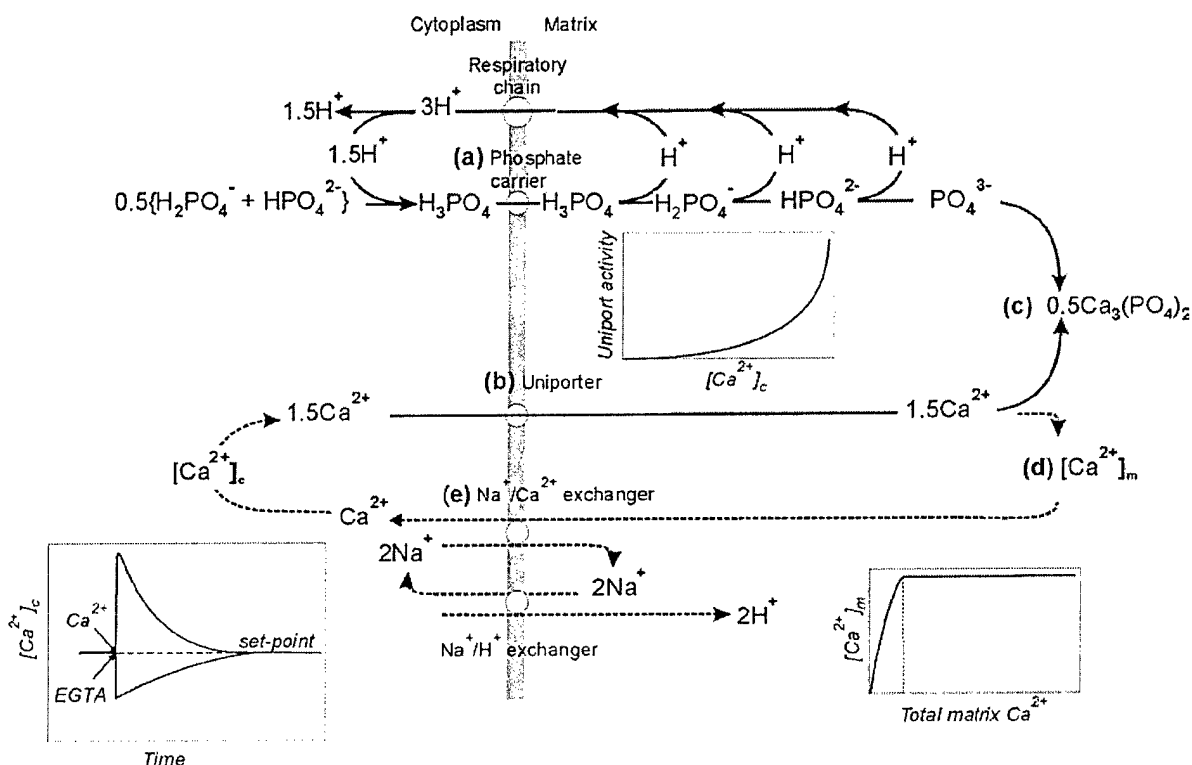


Fig. 1: Schematic of the ion movements involved in the net accumulation (solid arrows) and steady-state cycling (dashed arrows) of Ca^{2+} . (a) The phosphate carrier transports $H_2PO_4^-$ in exchange for OH^- but this is formally equivalent to the electroneutral transport of H_3PO_4 . Because 3 proton dissociations are required to form PO_4^{3-} the concentration of this species is inversely proportion to the cube of the proton concentration in the matrix (Chalmers and Nicholls 2003). (b) The uniporter activity increases as the 2.5 power of cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_c$ (Zoccarato and Nicholls 1982), see insert. (c) The tricalcium phosphate complex forms when its ion activity product is exceeded. Because the concentration of PO_4^{3-} increases with pH, the solubility of Ca^{2+} decreases and is about $2 \times 10^{-6} M$ when matrix pH is about 7.7 and external total phosphate is about 5mM (Chalmers and Nicholls 2003). (d) The matrix free Ca^{2+} concentration, $[Ca^{2+}]_m$, varies with total matrix Ca^{2+} until about 10nmol/mg is accumulated and the tricalcium phosphate complex start to form. In this initial region matrix Ca^{2+} can regulate tricarboxylic acid enzymes. Once the complex forms, $[Ca^{2+}]_m$ is invariant with matrix Ca^{2+} load and the cytoplasmic Ca^{2+} buffering mode is seen



(see insert). (e) The $\text{Na}^+/\text{Ca}^{2+}$ exchanger is controlled by $[\text{Ca}^{2+}]_m$; when the matrix is in cytoplasmic buffering mode ($>10\text{nmol Ca}^{2+}/\text{mg}$ accumulated) mitochondria seek to accumulate (or release) matrix Ca^{2+} to restore a set-point at which the kinetics of uptake via the uniporter exactly balance efflux via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Nicholls 1978).

In theory, both acetate and phosphate could serve as compensatory permeant anions to prevent the build-up of ΔpH and drop in $\Delta\Psi$; however their effects were distinct. With acetate the set-point rose slowly with Ca^{2+} load (Nicholls 1978; Zoccarato and Nicholls 1982) whereas with additional phosphate as permeant anion the set-point remained remarkably constant as total Ca^{2+} was varied from 10 to several hundred nmol/mg (Nicholls and Scott 1980). As investigated by Rossi and Lehninger (1964) matrix Ca^{2+} in the presence of phosphate appears to form some type of osmotically inactive complex, whereas calcium acetate is soluble. This raised the possibility that the remarkable invariance of the set-point over the range from 10-500nmol/mg Ca^{2+} (Chalmers and Nicholls 2003) was due to the kinetic balance between the activity of the uniporter at a given $[\text{Ca}^{2+}]_e$ and an activity of the efflux pathway that was essentially independent of total matrix Ca^{2+} load over this range because the free matrix Ca^{2+} , $[\text{Ca}^{2+}]_m$, was buffered at a constant value by the supposed Ca^{2+} -phosphate complex.

This hypothesis was tested in a subsequent study (Zoccarato and Nicholls 1982). Without making any assumptions about the chemical nature of the Ca^{2+} -phosphate complex, a constant ion activity ("solubility") product should mean that $[\text{Ca}^{2+}]_m$ would decrease when the matrix free phosphate concentration increased. This latter is surprisingly easy to control. Because the dominant pathway for P_i transport across the inner membrane is the electroneutral phosphate transporter (Palmieri *et al.* 1996), then if pH does not change, the concentrations of the different ionized forms of P_i free in the matrix will be proportional to the external P_i concentration. This was tested directly by monitoring the net rate of Ca^{2+} efflux from liver mitochondria following addition of ruthenium red to inhibit the uniporter. Ca^{2+} efflux rates decreased from 7nmol/min/mg protein from mitochondria phosphate depleted by glucose/hexokinase to 0.5nmol/min/mg protein in the presence of 3mM external P_i (Zoccarato and Nicholls 1982). Mitochondrial membrane potential did not change when P_i was increased, but the set-point decreased from 0.78 μM to 0.55 μM .

The rather modest change in set-point accompanying a 16-fold decrease in efflux rate indicates a very steep dependency of the uniporter on $[\text{Ca}^{2+}]_e$. This was investigated directly by an experiment in which Ca^{2+} was infused into mitochondrial incubations at varying rates and $[\text{Ca}^{2+}]_e$ was monitored until it achieved a constant value, implying that the net rate of Ca^{2+} accumulation by the mitochondria equalled the rate of the infusion and hence that the activity of the uniporter was equal to the infusion rate plus the activity of the efflux pathway. By varying the infusion rate the activity of the uniporter was determined to vary as the 2.5th power of $[\text{Ca}^{2+}]_e$. The contrast between this high dependency for uptake and the virtual independency of the efflux pathway on matrix Ca^{2+} load (due to the buffering of $[\text{Ca}^{2+}]_m$) is sufficient to account for the remarkable constancy of the set-point over this 50-fold range of total matrix Ca^{2+} from 10-500nmol/mg.

It should be emphasized in these studies that care was taken to reproduce the physiological conditions existing in the cytoplasm as accurately as possible, and in particular including adenine nucleotides in the incubation media. As evidenced from the literally thousands of publications on the permeability transition (PT), it is all too easy to incubate mitochondria in sucrose under non-physiological conditions, omit the natural protective action of exogenous adenine nucleotides, subject the unfortunate organelles to a massive bolus of Ca^{2+} and watch them swell. This is not to denigrate carefully controlled experiments designed to reproduce oxidative stress in, for example cardiac reperfusion models (Crompton 1999), but rather to emphasize the importance of realizing



that mitochondria operate in a controlled environment.

We have recently revisited the question of the nature of the matrix stored Ca^{2+} (Chalmers and Nicholls 2003) in order to answer a number of outstanding questions. In particular we were interested in confirming that the Ca^{2+} -phosphate complex in the matrix was able to maintain $[\text{Ca}^{2+}]_m$ at a constant value independent of total Ca^{2+} load and attempting to determine the factors that control the maximal Ca^{2+} loading capacity of the matrix. We were concerned about the bioenergetic loads associated with conventional bolus additions of the cation which result in a sudden demand on the proton gradient, transient depolarization, increased respiration and changes in ΔpH . Because each of these parameters changes rapidly during a bolus addition it is difficult to distinguish the precise factors that are for example responsible for defining the maximal capacity of the matrix to retain Ca^{2+} . Instead we adapted the infusion technique discussed above in order slowly to load the matrix with Ca^{2+} with negligible consequences for the mitochondrial bioenergetics

Liver mitochondria in the presence of ADP and oligomycin were able to accumulate 800nmol Ca^{2+} /mg over a period of 10min before $[\text{Ca}^{2+}]_e$ rose precipitously indicating the onset of a PT. No change in mitochondrial membrane potential occurred during the infusion until the onset of the transition. Some increase in NAD(P)H fluorescence was noted, together with an increase in light-scattering. It should be emphasized in this context that what is usually referred to as 'swelling' is actually measured as a decrease in light scattering caused by a decrease in the difference in refractive index between the matrix and the medium as the latter enters and dilutes the former. The increased light scattering during matrix Ca^{2+} loading is most likely due to the light-scattering properties or increased matrix refractive index due to the formation of the matrix Ca^{2+} -phosphate complex. Importantly, during the Ca^{2+} loading no increase in the level of reactive oxygen species could be detected, if anything H_2O_2 production decreased during Ca^{2+} loading (Chalmers and Nicholls 2003).

The PT inhibitor cyclosporin A enhances the Ca^{2+} loading capacity of liver mitochondria in the presence of ADP by 2.5-fold, while oxidation of endogenous NADH by the addition of acetoacetate decreases the loading capacity by a similar extent (Chalmers and Nicholls 2003). Interestingly acetoacetate is still effective in lowering loading capacity in the presence of cyclosporin A and ADP.

The nature of the Ca^{2+} -phosphate complex and its ability to buffer $[\text{Ca}^{2+}]_m$ that was predicted by the earlier studies on the set-point were investigated by loading rat brain mitochondria with the low affinity fura2-FF. As the total matrix load was varied either by the addition of Ca^{2+} or EGTA it became apparent that there was a discontinuity at about 10nmol total Ca^{2+} /mg protein. Above this value $[\text{Ca}^{2+}]_m$ was virtually invariant with total load, whereas below 10nmol/mg $[\text{Ca}^{2+}]_m$ varied as a linear function of total Ca^{2+} . This resolves a longstanding debate between advocates of matrix Ca^{2+} as a means of transmitting hormonal and metabolic changes in cytoplasmic Ca^{2+} into the matrix (Hansford 1994; McCormack and Denton 1990) and those including ourselves who had emphasized the Ca^{2+} buffering function of the mitochondrion (Nicholls and Åkerman 1982). It is apparent that the mitochondrion is beautifully adapted to either role. When $[\text{Ca}^{2+}]_e$ is maintained below the set-point there is too little matrix Ca^{2+} to form the Ca^{2+} -phosphate complex and $[\text{Ca}^{2+}]_m$ varies with $[\text{Ca}^{2+}]_e$, allowing for a messenger role for the cation in the control of the citric acid cycle. When however $[\text{Ca}^{2+}]_e$ rises, even briefly, above the set-point then sufficient matrix loading occurs for the formation of the Ca^{2+} -phosphate complex.

Studies with fluorescent Ca^{2+} indicators using either isolated mitochondria (Moreno and Hansford 1988; Davis *et al.* 1987; Lukacs and Kapus 1987; McCormack *et al.* 1989; Al Nasser and Crompton 1986) or intact cells (numerous studies employing rhod-2 and related indicators) have reported



surprisingly low values for $[Ca^{2+}]_m$ - in the range 1-5 μM under a variety of conditions. Indeed in the regulatory range (0-10 nmol total Ca^{2+} /mg) the effects on citric acid cycle enzymes are consistent with $[Ca^{2+}]_m$ values of 0.5-2 μM (Hansford and Castro 1982). In our study $[Ca^{2+}]_m$ for brain mitochondria remained in the range 2-3 μM when total Ca^{2+} was increased from 10 to 500 nmol/mg (Chalmers and Nicholls 2003). Thus the earlier prediction that $[Ca^{2+}]_m$ should be essentially independent of total matrix Ca^{2+} (Zoccarato and Nicholls 1982) was confirmed. Furthermore the expected inverse relationship between external P_i concentration and $[Ca^{2+}]_m$ was also found. Interestingly cyclosporin A, although it more than doubled the maximal loading capacity of the matrix did not affect the stability of the Ca^{2+} -phosphate complex.

A major puzzle has been how to reconcile the apparent properties of the matrix Ca^{2+} -phosphate complex with that of known complexes in solution. Physiological cell incubation media contain millimolar Ca^{2+} in solution in the presence of millimolar P_i , and yet in the matrix some form of osmotically inactive complex forms when $[Ca^{2+}]_m$ rises above 1-5 μM . Furthermore, *in vitro* calcium phosphate complexes once formed are notoriously difficult to redissolve, whereas the addition of a protonophore to Ca^{2+} -loaded mitochondria leads to an extremely rapid efflux of Ca^{2+} via reversal of the uniporter and phosphate separately via the phosphate transporter (Zoccarato and Nicholls 1982). Finally, if the gradient of free Ca^{2+} across the inner membrane is really so low (say 0.5 μM outside and 2 μM inside), as is the gradient of free P_i (defined by the low ΔpH) why is the addition of protonophore so effective in triggering massive and rapid Ca^{2+} release?

It is not possible to investigate the nature of the matrix Ca^{2+} -phosphate complex directly, due to its instant dissociation when the mitochondria are disrupted and to the ability of Ca^{2+} -phosphate complexes to change during fixation or drying for physicochemical analysis. Thus hydroxyapatite can be detected in fixed and desiccated samples, but it is generally accepted that this is an artifact. In an artificial cytoplasm in the presence of ATP, amorphous $Ca_3(PO_4)_2$ is initially formed when millimolar Ca^{2+} is titrated in, particularly at alkaline pH (Wuthier *et al.* 1985).

Some limits on the stoichiometry of the matrix complex can be reached by accurate determination of the ratio of Ca^{2+} accumulated to net protons extruded during the uptake. These classic studies by Lehninger and colleagues produced values close to 1.0 H^+/Ca^{2+} (Lehninger *et al.* 1967). While it was not initially realized that this was a 'chemiosotic' proton extruded by the respiratory chain the analysis remains valid. This ratio is consistent with the formation of $Ca_3(PO_4)_2$ (Fig. 1) whereas hydroxyapatite with a formula $Ca_5(PO_4)_3OH$ would give a ratio of 1.1. While these ratios are perhaps too close to allow unambiguous discrimination, other forms such as $CaHPO_4$ (ratio 0.5) and $Ca(H_2PO_4)_2$ (ratio -1) can be eliminated.

The clue both to the low Ca^{2+} solubility in the matrix and the rapid efflux initiated by protonophore appears to lie in the pH gradient across the inner membrane. The highly active phosphate carrier equilibrates the transported species $H_2PO_4^-$ with OH^- and thus accumulates the anion as a function of the ΔpH . Two further dissociations of $H_2PO_4^-$ to HPO_4^{2-} and PO_4^{3-} are required before $Ca_3(PO_4)_2$ is formed and both of these dissociations are dependent on the matrix pH, with the final result that the concentration of the PO_4^{3-} species is dependent on the third power of the ΔpH at constant external phosphate.

The solubility of a salt is governed by the ion activity product (solubility product). Values reported in the literature for the calcium phosphate complexes are somewhat variable, but values of 3×10^{-30} for amorphous $Ca_3(PO_4)_2$ and 1×10^{-59} for hydroxyapatite are representative. From the pK 's for the dissociation of the phosphate anionic forms it can be calculated that the trans-membrane gradient of the PO_4^{3-} anion varies as the third power of the pH gradient, so that an increase in matrix pH from



7 to 8 would increase the matrix PO_4^{3-} concentration by 1000-fold. Since the ion activity product is a constant, this means that taking an example of mitochondria incubated in the presence of 5mM total external Pi, the saturating Ca^{2+} concentration in equilibrium with tri-calcium phosphate in the matrix would decrease from about 100 μM at pH 7 to 1 μM at pH 8, while the corresponding saturating Ca^{2+} concentrations in equilibrium with hydroxyapatite would be about 15-fold lower (Chalmers and Nicholls 2003).

The best fit with the experimental data for $[\text{Ca}^{2+}]_m$ would be consistent with tri-calcium phosphate in the matrix of respiring mitochondria at a pH of about 7.7, when saturating $[\text{Ca}^{2+}]_m$ would be calculated to be in the region of 2 μM . Thus the low values reported for $[\text{Ca}^{2+}]_m$ are entirely consistent with the physical chemistry of calcium phosphate complexes at alkaline pH. This also provides an explanation for the rapid efflux of Ca^{2+} and Pi on their respective carriers when protonophores are added, since this is associated with a dramatic matrix acidification. This would decrease the concentration of PO_4^{3-} , destabilizing the complex and increasing $[\text{Ca}^{2+}]_m$ to about 100 μM , the high Ca^{2+} gradient thus driving the rapid efflux of the cation.

The Ca^{2+} -transport properties of isolated mitochondria are sufficient to account for the major features observed for *in situ* mitochondrial Ca^{2+} transport. In particular mitochondria only appear to sequester significant amounts of Ca^{2+} when $[\text{Ca}^{2+}]_m$ rises above 0.5 μM , corresponding rather nicely with the set-point observed for isolated mitochondria (Werth and Thayer 1994). Isolated mitochondria release Ca^{2+} when the external Ca^{2+} concentration falls below the set-point (Nicholls 1978) and this behavior can be observed in cultured neurons recovering after a transient cytoplasmic Ca^{2+} load, where the recovery to basal $[\text{Ca}^{2+}]_c$ is delayed by a shoulder consistent with an unloading of the temporarily accumulated cation to the cytoplasm (Werth and Thayer 1994).

In conclusion, the mitochondrial Ca^{2+} transport and sequestration properties are perfectly adapted to accumulate, store and release large amounts of the cation. The pioneering work of Al Lehninger and his colleagues laid the essential ground-work from which these studies have evolved.

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Mitochondrial trafficking in neurons: a key variable in neurodegeneration?

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Abstract

Mitochondria are the proximate target of a number of different neurotoxins. Typically, one considers the action of toxins to impair the key bioenergetic function of mitochondria as the main mechanism of action. However, the effective maintenance of energy generation in neurons depends on the biogenesis, trafficking and degradation of mitochondria in addition to the traditional bioenergetic functions. We have recently demonstrated that glutamate alters both the trafficking and morphology of mitochondria in primary neurons. In addition, several other potential neurotoxins including nitric oxide and zinc inhibit mitochondrial movement and, in some cases alter morphology too. This suggests that some part of the action of neurotoxins might include the impairment of mitochondrial trafficking in neurons, with the resultant failure of local ATP delivery.

Introduction

Mitochondria have emerged as key targets of a number of different types of neurotoxin. In excitotoxic neuronal injury, activation of N-methyl-D-aspartate (NMDA) receptors activates a massive calcium entry that is initially buffered by mitochondria (Budd and Nicholls, 1996; White and Reynolds, 1995). However, the mitochondrial calcium loading disrupts mitochondrial function by depolarizing the mitochondrial membrane potential ($\Delta\Psi_m$) (Khodorov et al., 1996; Schinder et al., 1996; White and Reynolds, 1996), stimulating oxidant production (Dugan et al., 1995; Reynolds and Hastings, 1995), and causing cytochrome c release (Budd et al., 2000). Which of these events, if any, kill neurons in excitotoxicity is unclear, but preventing mitochondrial calcium accumulation effectively protects neurons from injury (Budd et al., 2000; Stout et al., 1998). Mitochondria are also the target of a number of neurotoxins that work more slowly than NMDA-mediated excitotoxicity. Toxins that target complex I of the electron transport chain, such as MPP⁺ and rotenone, cause injury closely resembling Parkinson's disease in rodent models (Betarbet *et al.*, 2000; Singer *et al.*, 1987). Systemic administration of 3-nitropropionic acid, a complex II poison, selectively kills neurons in the striatum, and produces a syndrome like Huntington's disease {ref}. Again, the critical impairment responsible for committing neurons to die following exposure to these toxins is not clear. However, a combination of energetic impairment and enhanced production of oxidants may well account for the injury.

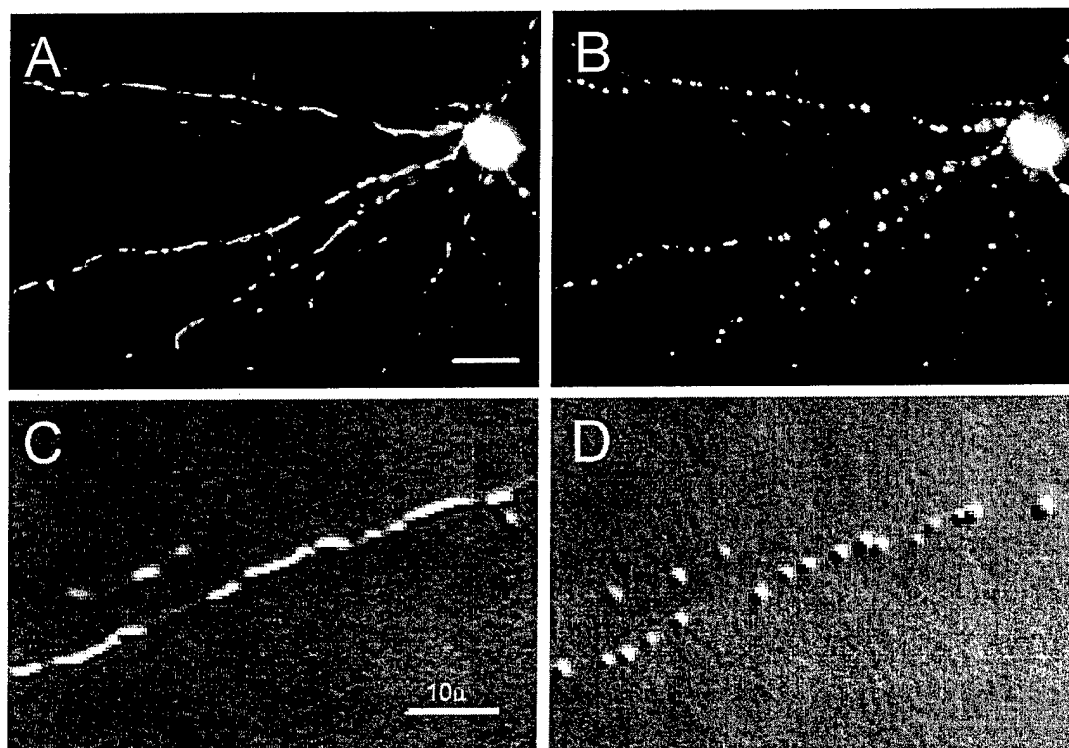


Figure 1. Glutamate induced changes in mitochondrial morphology. These images were obtained from a neuron in primary culture. The neuron expressed mitochondrially targeted enhanced yellow fluorescent protein. Panel A shows mitochondria before, while panel B was taken after, a 10 minute exposure to 30μM glutamate. Panels C and D show the same effect with enhancement of the image to illustrate that fragmentation occurs in addition to the swelling of the mitochondria.

In attempting to link mitochondrial failure to neuronal injury, the range of mitochondrial dynamics to consider in neurons extends well beyond simply ATP and oxidant production. In all cells, effective mitochondrial function depends on an intact mitochondrial life history, from biogenesis through delivery of mitochondria to appropriate cellular targets and ultimately the correct retrieval and degradation of the organelles at the end of their effective lifetime. Although the details of mitochondrial life history in neurons have not been clearly established, it is easy to appreciate the additional challenges faced by neurons because of the distances over which mitochondria must travel in order to supply ATP to regions of the cell that have high energy demands. An additional variable is that of mitochondrial morphology. It has been appreciated for some time that the shape of mitochondria varies considerably between cell types. Intriguingly, recent studies have suggested that morphology is a key variable of mitochondria within cells, and one that may govern the outcome from injury (Frank *et al.*, 2001; Karbowski and Youle, 2003). Here we will review recent data demonstrating that both movement and morphology of mitochondria are affected by neurotoxins.

Neuronal Injury Changes Mitochondrial Morphology

Several types of morphological change in mitochondria can be anticipated based on current knowledge. Perhaps the most obvious is the swelling that should be associated with the induction of permeability transition, an event associated with excitotoxic neuronal injury (Reynolds and



Hastings, 2001; Schinder *et al.*, 1996). In addition, the regulation of mitochondrial fission and fusion can result in a net alteration in mitochondrial shape, and it has been proposed that fission of mitochondria is associated with apoptotic cell injury (Frank *et al.*, 2001). We recently described effects of glutamate on the morphology and movement in neurons in culture (Rintoul *et al.*, 2003). Acute application of glutamate and the subsequent influx of calcium through NMDA receptors results in a cessation of movement (discussed further below), a profound alteration in morphology and also an occasional fragmentation of mitochondria (Figure 1) that occurs within minutes of glutamate stimulation. The change in morphology is calcium dependent, and is associated with a disruption of the cytoskeleton. This morphological change could be attributed to mitochondrial swelling. However, the lack of sensitivity to cyclosporin A makes it difficult to conclude that permeability transition is the cause of the swelling. Interestingly, the glutamate-induced morphological change recovers within 1-2 hours, well before neurons die from this stimulus.

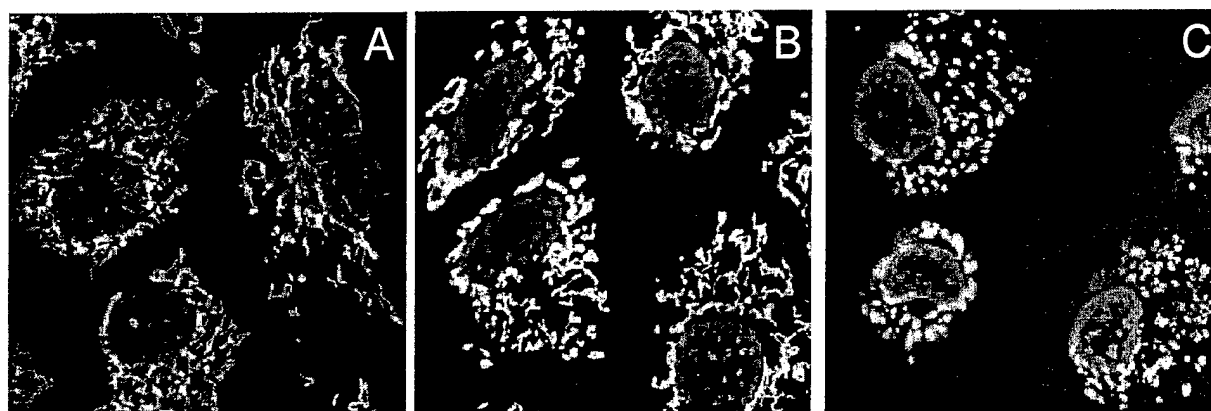


Figure 2. Zinc alters mitochondrial morphology. This experiment was performed in HT22 cells that were stably transfected with mt-eYFP (green fluorescence) and also stained with Hoechst 33342 to label the nucleus. Panel A shows control cells, while the cells in B and C were exposed to 3 μ M zinc in the presence of 20 μ M pyrithione. Panel B was imaged 1hr after zinc, while panel C was obtained after 2hr. Note the loss of the filamentous structure of the mitochondria as a consequence of the zinc treatment.

Zinc is an important endogenous neurotoxin that is mobilized during ischemic brain injury (Koh *et al.*, 1997). We have recently found that zinc produces a different form of morphological change in mitochondria. We exposed HT22 cells that express mitochondrially-targeted enhanced yellow fluorescent protein (mt-eYFP) to zinc in the presence of an ionophore, pyrithione. Over the next two hours, zinc exposure resulted in a progressive fragmentation of mitochondria that precedes loss of cytochrome c or nuclear condensation (Figure 2). This indicates that cytochrome c release is not simply a consequence of the mitochondria being shredded during the execution of apoptosis. The molecular basis for the fragmentation is not clear, but it has been shown that cells can be protected from apoptosis with a dominant negative form of the fission promoting protein DRP1 (Frank *et al.*, 2001), and it is tempting to speculate that this response reflects an induced imbalance between mitochondrial fission and fusion that makes an important contribution to the fate of the cell.

Neuronal Injury Changes Mitochondrial Movement.

As already noted, neuronal mitochondria may have to travel considerable distances to reach the site of ATP demand. Processes that disrupt the delivery of mitochondria may negatively impact



neuronal viability by essentially imposing a local starvation because of the absence of necessary mitochondria. We have recently found a number of conditions that impair mitochondrial movement. The first of these was NMDA receptor activation with glutamate. As described above, glutamate alters mitochondrial morphology, but also produces a profound inhibition of mitochondrial movement (Rintoul *et al.*, 2003). In an effort to determine potential mechanisms for this effect, we compared the actions of glutamate to the uncoupler FCCP, which depolarizes $\Delta\Psi_m$, and oligomycin, which inhibits ATP synthesis. Both of these agents inhibited movement, but did not alter morphology. This suggests that movement critically depends on ATP production by mitochondria, and further implies that the morphological change is a consequence of the calcium entry produced by glutamate but not FCCP or oligomycin (Figure 3).

The latter conclusion suggested an additional series of experiments. It has been well established that nitric oxide is an effective inhibitor of complex IV of the electron transport chain at relatively low concentrations [ref]. We speculated that NO would impair mitochondrial movement. Exposing neurons to the NO donor PAPA nonoate confirmed this to be the case, and NO appears to decrease movement without altering mitochondrial morphology. We have found that several other neurotoxins decrease mitochondrial movement in neurons, including elevated intracellular zinc and oxidant exposure, but the mechanisms by which these effects occur are still under investigation.

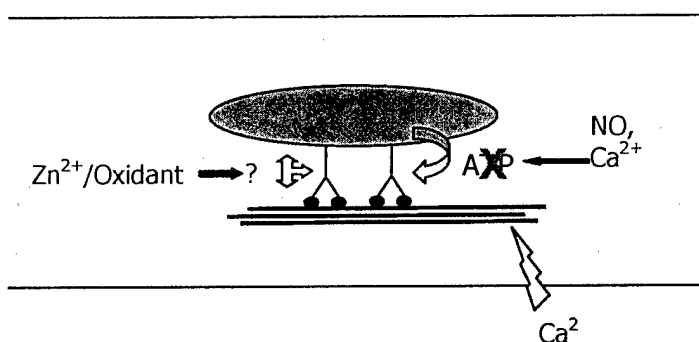


Figure 3. Mechanisms for altering mitochondrial movement in neurons. There are likely to be three or more mechanisms for altering trafficking. Calcium can act by disrupting cytoskeletal structures like microtubules. Agents like NO and calcium can also impair movement by inhibiting ATP synthesis by mitochondria. Other neurotoxins, including zinc and some oxidizing agents inhibit mitochondrial movement without obviously depolarizing mitochondria, so this is likely to be the result of a separate mechanism.

Conclusions

These studies illustrate the point that neurotoxins can acutely alter mitochondrial movement and morphology in addition to the known effects on ATP synthesis and oxidant generation. It seems likely that inhibiting movement will result in the impairment of the delivery of mitochondria to relevant sites within neurons, although it remains to be established whether there is a specific link between the cessation of movement and injury to neurons. It should also be noted that nerve growth factor inhibits mitochondrial movement in peripheral neurons (Chada and Hollenbeck, 2003). Nerve growth factor would not be considered neurotoxic in these cells, and the transient docking of mitochondria proposed as an action of the trophic factor might be considered to be a beneficial effect. It is more difficult to discern the consequence of morphological alterations for the function of neuronal mitochondria. Gross swelling as a consequence of calcium overload is probably a reflection of mitochondrial impairment, rather than a specific indicator of altered function. The more subtle fragmentation presumably yields mitochondria that are functionally intact, so that the specific



hazard resulting from the decrease in mitochondrial size remains to be elucidated. However, it does raise the interesting possibility that the mechanisms that control fission or fusion might represent a novel target for neuroprotection.

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Deadly Conversations: Nuclear-Mitochondrial Cross-Talk

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Abstract

Neuronal damage following stroke or neurodegenerative diseases is thought to stem in part from overexcitation of N-methyl-D-aspartate (NMDA) receptors by glutamate. NMDA receptors triggered neurotoxicity is mediated in large part by activation of neuronal nitric oxide synthase (nNOS) and production of nitric oxide (NO). Simultaneous production of superoxide anion in mitochondria provides a permissive environment for the formation of peroxynitrite (ONOO⁻). Peroxynitrite damages DNA leading to strand breaks and activation of poly(ADP-ribose) polymerase-1 (PARP-1). This signal cascade plays a key role in NMDA excitotoxicity, and experimental models of stroke and Parkinson's disease. The mechanisms of PARP-1 mediated neuronal death are just being revealed. While decrements in ATP and NAD are readily observed following PARP activation, it is not yet clear whether loss of ATP and NAD contribute to the neuronal death cascade or are simply a biochemical marker for PARP-1 activation. Apoptosis-inducing factor (AIF), is normally localized to mitochondria but following PARP-1 activation, AIF translocates to the nucleus triggering chromatin condensation, DNA fragmentation and nuclear shrinkage. Additionally, phosphatidylserine is exposed and at a later time point cytochrome c is released and caspase-3 is activated. In the setting of excitotoxic neuronal death, AIF toxicity is caspase independent. These observations are consistent with reports of biochemical features of apoptosis in neuronal injury models but modest to no protection by caspase inhibitors. It is likely that AIF is the effector of the morphologic and biochemical events and is the commitment point to neuronal cell death, events that occur prior to caspase activation, thus accounting for the limited effects of caspase inhibitors. There exists significant cross talk between the nucleus and mitochondria, ultimately resulting in neuronal cell death. In exploiting this pathway for the development of new therapeutics, it will be important to block AIF translocation from the mitochondria to the nucleus without impairing important physiological functions of AIF in the mitochondria.

Introduction

The extent and cost of neurologic disease is staggering. 50 million Americans have a permanent, neurological disability that limits their daily activities. Chronic neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and Lou Gehrig's disease (ALS) afflict over 6.5 million Americans. Every 53 seconds someone in the USA suffers a stroke, affecting over 3 million Americans each year with over 4.4 million stroke survivors who have significant disability. Clearly new strategies need to be developed to treat these patients. Basic fundamental research in cell signaling and biochemistry, have begun to identify the key elements of the brain and nervous system that mediate neuronal injury. Identifying these signaling molecules will open the door to new clinical opportunities and have the potential to impact millions of lives.

Neurotoxicity

Many pathways have been proposed for neuronal damage in stroke and neurodegenerative diseases including extrinsic and intrinsic apoptotic programs and excitotoxicity. Glutamate excitotoxicity is a common finding that is mediated by intracellular calcium, nitric oxide and free radicals (Dirnagl *et al.* 1999; Kristian and Siesjö 1998; Lipton 1999). Glutamate initiates its actions



postsynaptically by binding to four major types of receptors: metabotropic receptors, N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and kainate receptors (Mayer and Westbrook 1987). NMDA receptor activation mediates, in large part, glutamate excitotoxicity and neuronal damage. Glutamate stimulated NMDA receptors flux calcium and activate a variety of intracellular calcium-dependent enzymes and processes, of which, activation of neuronal NO synthase (nNOS) plays a prominent role (Samdani *et al.* 1997). Thus, overproduction of NO from excessive or inappropriate stimulation of nNOS appears to mediate a major component of excitotoxic damage although other reactive oxygen species are also generated in excitotoxic conditions.

Reactive Oxygen Species (ROS)

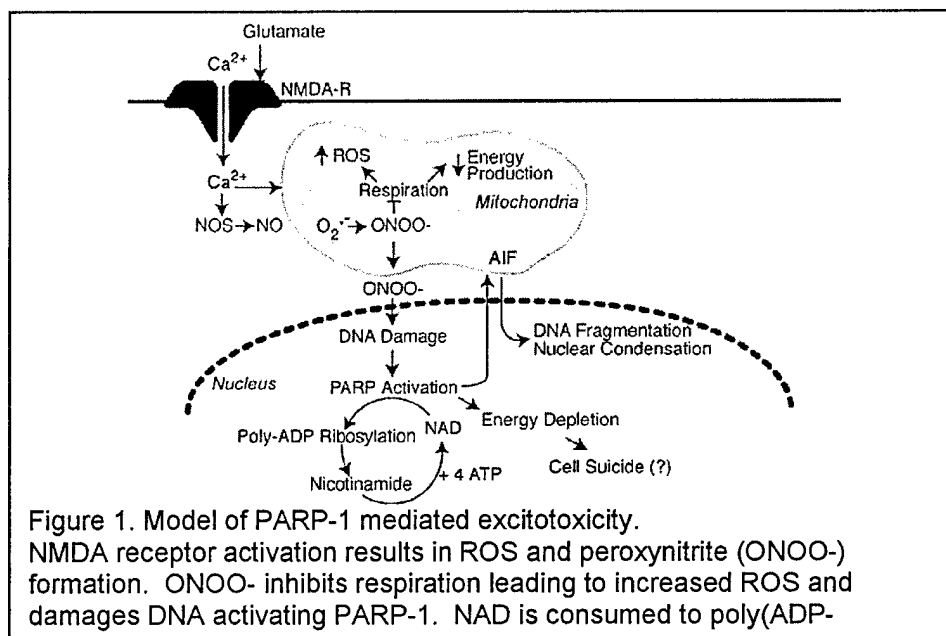
The inherent biochemical and physiological characteristics of the brain, including high lipid concentrations and energy requirements, make it particularly susceptible to free radical mediated insult. When oxygen free radicals are generated in excess of a cell's antioxidant capacity, severe damage to cellular constituents including proteins, DNA and lipids can occur (Chan 2001). The oxygen species that are typically linked to oxidative stress are superoxide anion, hydroxyl radical ($^{\bullet}\text{OH}$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite (ONOO^-). In mitochondria, generation of these species from molecular oxygen is a normal aspect of mammalian respiration, and over activation of the NMDA receptor results in the increased formation of reactive oxygen species (Chan 2001). NMDA receptor-mediated stimulation of phospholipase A_2 and the subsequent release of arachidonic acid, prostaglandins, leukotrienes, thromboxanes and platelet-activating factor leads to a variety of toxic events including generation of oxygen free radicals (Dimagli *et al.* 1999). These processes can cause the neuron to digest itself by protein breakdown, free-radical formation, and lipid peroxidation. Under conditions of calcium elevation and energy failure, xanthine dehydrogenase is converted to xanthine oxidase, the activity of which results in superoxide anion formation (Chan 2001). The superoxide anion is generated by multiple pathways and is often placed at the start of an oxidative stress cascade. The brain derives most of its energy exclusively from oxidative respiration through the mitochondrial electron transport chain. Mitochondria are located throughout the neuronal perikarya and its processes. During the production of ATP there is a small high-energy electron "leak" (1-3%) resulting in the generation of superoxide anion. Superoxide anion is constrained by membranes that it cannot cross and is retained within mitochondria. Superoxide dismutase (SOD) catalyzes the conversion of superoxide anion to H_2O_2 , which is diffusible within and between cells (Chan 2001). NO is synthesized on demand by the enzyme NOS from the essential amino acid, L-arginine (Dawson and Dawson 1998). There are three NOS genes, neuronal NOS (nNOS), endothelial NOS and immunologic NOS that were named by the tissue from which they were first cloned. NO is small, diffusible, membrane permeable and reactive. The biochemical reactions involving NO are not well characterized. Probably the most important oxidant involved in the genesis of neurotoxicity is peroxynitrite (ONOO^-). Peroxynitrite is formed from the reaction of NO with superoxide anion. *In vitro* the rate of this reaction is three times faster than the rate of reaction of the enzyme, superoxide dismutase (SOD), in catalyzing the dismutation of superoxide anion (Ischiropoulos and Beckman 2003). Therefore, when present at appropriate concentrations, NO can effectively compete with SOD for superoxide anion. Although a simple molecule, peroxynitrite is chemically complex. It has the activity of hydroxyl radical and nitrogen dioxide radical, although it does not readily decompose into these entities. Peroxynitrite can also directly nitrate and hydroxylate aromatic rings on amino acid residues. It is also a potent oxidant that reacts readily with sulfhydryls, with zinc-thiolate, lipids, proteins and DNA (Ischiropoulos and Beckman 2003).



Poly (ADP-Ribose) Polymerase (PARP)

The discovery that inhibitors of PARP are neuroprotective against NMDA and NO neurotoxicity initiated interest in PARP activity in the CNS (Eliasson *et al.* 1997; Endres *et al.* 1997; Zhang *et al.* 1994). PARP-1 is a member of a growing family of proteins that now included 18 putative PARP proteins based on protein domain homology and enzymatic function. PARP-1 is the PARP responsible for large branch chain polymers and generates >95% of the poly(ADP-ribose) in a cell. The obligatory trigger of PARP-1 activation is DNA strand nicks and breaks, which can be induced by a variety of environmental stimuli and free radical/oxidant attacks, including oxidants (hydrogen peroxide, hydroxyl radical, peroxynitrite), ionizing radiation, and genotoxic agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). In response to DNA damage, PARP-1 becomes activated and, using NAD⁺ as a substrate, it builds up polymers of adenosine diphosphate ribose (de Murcia and Menissier de Murcia 1994; Lindahl *et al.* 1995). Poly(ADP-ribose) acceptors include histones, topoisomerases I and II, DNA polymerases and DNA ligase 2, as well as PARP-1 itself. Poly-ADP-ribosylation might result in an inhibition of the activity of some of these enzymes. In the case of histones, poly-ADP-ribosylation stimulates chromatin relaxation. The physiological function of PARP-1 and poly (ADP-ribose) is still under heavy debate. From studies using pharmacological inhibitors of PARP-1, poly(ADP-ribose) has been suggested to regulate gene expression and gene amplification, cellular differentiation and malignant transformation, cellular division and DNA replication, as well as apoptotic cell death (Chiarugi 2002).

Pharmacologic inhibition of PARP-1 or genetic knockout of PARP-1 elicits cytoprotection in a variety of disease models including ischemia-reperfusion injury, diabetes, inflammatory-mediated injury, reactive oxygen species-induced injury, glutamate excitotoxicity and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injury (Eliasson *et al.* 1997; Endres *et al.* 1997; Mandir *et al.* 1999; Szabo and Dawson 1998; Yu *et al.* 2002; Zhang *et al.* 1994). How PARP-1 activation kills neurons is not known. A cell suicide hypothesis was developed in the 1980's (Berger and Berger 1986; Berger *et al.* 1983) and has been used to explain the actions of PARP-1 in the CNS (Figure 1.). The suicide theory is based on the role of cellular NAD⁺ to regulate an array of vital cellular processes. NAD⁺ serves as a cofactor for glycolysis and the tricarboxylic acid cycle, thus providing ATP for most cellular processes (Hageman and Stierum 2001). NAD⁺ also serves as the precursor





for NADP, which acts as a co-factor for the pentose shunt, for bioreductive synthetic pathways, and is involved in the maintenance of reduced glutathione pools (Hageman and Stierum 2001). The observation that activation of PARP-1 can lead to massive NAD⁺ utilization, and changes in the cellular NAD⁺ and ATP levels led Berger and Okamoto to propose that consumption of NAD⁺ due to DNA damage and activation of PARP-1 can affect cellular energetics and function ultimately leading to cell death due to excessive energy consumption (Berger and Berger 1986; Berger *et al.* 1983). Studies in various cell and animal models have consistently observed depletion of NAD⁺ and ATP that is blocked by PARP-1 inhibition or deletion. However, none of these studies address whether loss of cellular energetics is the cause of excitotoxic cell death or is simply a biomarker for PARP-1 activation. This is a difficult question to experimentally address. Addition of precursors such as creatine, can provide protection but these agents activate multiple mitochondrial and cellular pathways that may protect cells in a manner that is independent from the signal cascade triggered by PARP-1 mediated NAD⁺ loss. In an experimental stroke model recent data indicate that preservation of energy stores in PARP-1 knockout mice is not the mechanism underlying the reduction in infarct volume (Goto *et al.* 2002). The time-course and severity of the apparent diffusion coefficient (ADC), an *in vivo* measure of cellular energy stores, is not altered in PARP-1 knockout brains compared with wild-type brains, despite the fact that the PARP-1 knockout animals had smaller infarct volumes compared with wild type animals (Goto *et al.* 2002). Thus, energy depletion alone might not be sufficient to mediate PARP-1-dependent cell death. Recently we identified a role for AIF as a downstream signaling molecule in the PARP-1 dependent cell death but how PARP-1 triggers AIF release and cell death is not yet known (Figure 1).

Apoptosis Inducing Factor (AIF):

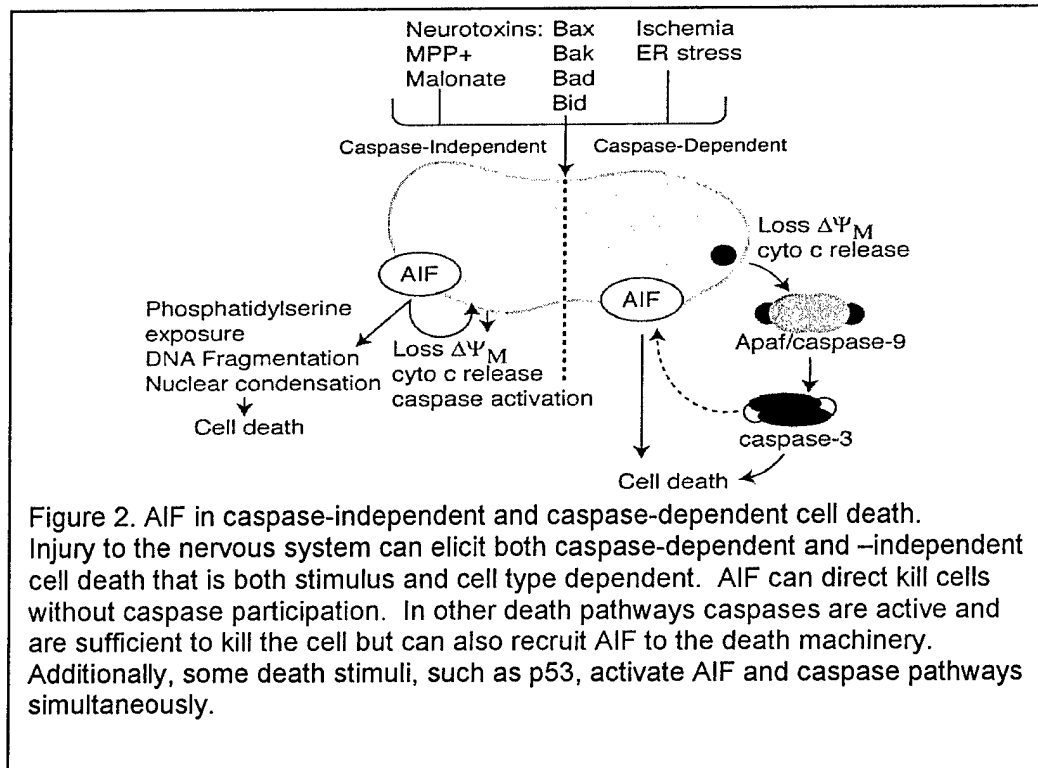
Recent and emerging data indicate that AIF plays an important role in excitotoxic neuronal death. Mammalian AIF is a 67 kD protein containing an N-terminal mitochondrial localization sequence and a large C-terminal with homology to bacterial oxidoreductases (Susin *et al.* 1999). AIF is evolutionarily conserved with homologs found in invertebrates, nematodes, fungi, and plants. AIF can stably bind FAD, which places AIF in the category of flavoproteins. AIF also displays NAD(P)H oxidase as well as monodehydroascorbate reductase activities (Miramar *et al.* 2001). The overall crystal structure of mature mouse AIF has been recently resolved at 2.0 Å resolution. AIF displays a glutathione-reductase-like fold, with an FAD-binding domain, an NADH-binding domain, and a C-terminal domain that bears a small AIF-specific insertion (509-559) not found in glutathione reductase. The amino acids interacting with FAD and NADH have been mapped precisely, and the mutants E313A and K176A have been shown to reduce FAD binding (Mate *et al.* 2002). Mutational analysis reveals that regardless of the presence or the absence of NAD(P)H and/or FAD (which is the essential prosthetic group of the oxidoreductase), AIF can induce nuclear apoptosis (Loeffler *et al.* 2001; Miramar *et al.* 2001). These data indicate that the oxidoreductase function of AIF is not required for its apoptogenic action.

The normal physiologic activity of AIF is not known. Recent data from the Harlequin mice in which expression of AIF 67 kD is reduced 80% due to a proviral insertion into AIF genomic DNA suggests that AIF might participate in scavenging ROS (Klein *et al.* 2002). The putative redox reaction catalyzed by AIF in mitochondria is also not known. Based on its similarity to prokaryotic oxidoreductases, it is possible that AIF might interact with the cytochrome bc₁ complex, which catalyzes the electron transfer from ubiquinone to cytochrome *c* in the mitochondrial respiratory chain (Mate *et al.* 2002). Theoretically, AIF could catalyze the reduction of cytochrome *c* in the presence of NADH *in vitro* (Miramar *et al.* 2001).

Following exposure of a cell to cytotoxic insults, AIF translocates to the cytosol and then the nucleus, where it induces peripheral chromatin condensation and high-molecular-weight (50 kb) DNA fragmentation. Translocation of AIF to the nucleus appears to be a general feature of apoptosis in mammalian cells (Cande *et al.* 2002) but whether it is a primary execution step or a



secondary participatory step is dependent on the death signal and cell type. Confirmation of the death effector role for AIF when released from its normal mitochondrial compartment, are the observations resulting from forced cytosolic expression of AIF in the absence of an external death signal (Loeffler *et al.* 2001). The crystal structure of human AIF revealed the presence of a strong positive electrostatic potential at the AIF surface. AIF co-localizes with DNA at an early stage of nuclear morphological changes, as indicated by electron microscopy. The electrostatic interaction between AIF and DNA is independent of the DNA sequence. Structure-based mutagenesis showed that DNA-binding defective mutants of AIF fail to induce cell death (Ye *et al.* 2002). This suggests that DNA binding by AIF is required for its apoptogenic function in the nuclear compartment. Two of the mutants that completely blocked the capacity of AIF to interact with DNA and to induce chromatin condensation (K255A, R265A and K510A/K518A), still retained NADH oxidase activity (Ye *et al.* 2002), thus confirming that the oxidoreductase and apoptosis-inducing activities of AIF can be fully dissociated. How AIF induces chromatin condensation and DNA fragmentation remains a mystery. There are several possible scenarios. AIF could itself have some cryptic nuclease activity that has not yet been observed. The interaction of AIF with DNA may increase the susceptibility of DNA to latent endogenous nucleases. AIF might recruit nucleases to induce partial chromatin fragmentation. The mitochondrial-nuclear translocation of AIF is caspase-independent in some types of cell death (Cregan *et al.* 2002; Susin *et al.* 1999; Yu *et al.* 2002) as treatment with caspase inhibitors fails to block AIF translocation and cell death (Yu *et al.* 2002). Additionally, translocation of AIF can be observed *in vitro* in cells in which there is no caspase activation, owing to genetic deletion of Apaf-1, caspase-9 or caspase-3 (Cregan *et al.* 2002; Susin *et al.* 2000). Similar observations have been obtained in *Apaf-1*^{-/-}, *caspase-9*^{-/-} or *caspase-3*^{-/-} embryoid bodies, in which AIF translocates during cavitation (Joza *et al.* 2001). Microinjection or transfection of *Apaf-1*^{-/-}, *caspase-9*^{-/-} or *caspase-3*^{-/-} cells with recombinant AIF protein or transient expression of AIF, also induces cell death without caspase activation. Features of classic apoptosis, such as phosphatidylserine exposure, partial chromatin condensation and cellular shrinkage are still observed in these cells (Loeffler *et al.* 2001; Susin *et al.* 2000). *In vitro*, both purified natural AIF and recombinant AIF alter the structure of chromatin resulting in large-scale DNA fragmentation in purified nuclei. This "nuclear apoptosis" cannot be prevented by caspase inhibitors (Susin *et al.* 2000; Yu *et al.* 2002). While these data strongly support the notion that AIF acts as a caspase independent death effector, there also exists data for an interaction between AIF and the caspase cascade. Activated caspases (caspase-8 and caspase-2) and the caspase-activated protein *t*-Bid can trigger the release of AIF from mitochondria (Lassus *et al.* 2002; Robertson *et al.* 2002; Zamzami *et al.* 2000). In HeLa and Jurkat cell lines treated with staurosporine or actinomycin D, mitochondrial release of AIF is suppressed or delayed by caspase inhibitors (Arnoult *et al.* 2001). Genetic data obtained in *C. elegans* also suggest that AIF operates partially in a caspase-dependent fashion. Heat-shock inducible expression of Egl-1 causes the mitochondrial release of green fluorescent protein (GFP)-tagged AIF in all *C. elegans* wild-type embryos, but *Ced-3* loss-of-function mutants there is a >80% inhibition of AIF release (Wang *et al.* 2002). Transgenic expression of AIF and endonuclease G kills >60% of wild-type cells in *C. elegans*, but in *Ced3*-deficient animals cell death is reduced to 28%. These data suggest that, although AIF and endonuclease G can function in a caspase-independent fashion, AIF and endonuclease G are more efficient in a *Ced-3*-positive background (Wang *et al.* 2002). Taken together the published literature supports both caspase-independent or caspase-dependent mechanisms of AIF release from the mitochondria that are dependent on cell type and the lethal stimulus (Figure 2).



In the CNS AIF may be particularly important in mediating neurotoxicity due to both acute and chronic (neurodegenerative) diseases. Acute neurotoxicity can be induced by trauma, hypoglycemia or transient ischemia. The translocation of AIF has been observed in several experimental models of neurotoxicity such as the death of photoreceptors induced by retinal detachment (Hisatomi *et al.* 2002), neuronal cell death induced *in vivo* by brain trauma (Zhang *et al.* 2002) and death of cortical neurons induced *in vitro* by exposure to heat-inactivated *Streptococcus pneumoniae* (Braun *et al.* 2001), hydrogen peroxide, peroxynitrite (Zhang *et al.* 2002), the topoisomerase I inhibitor camptothecin, infection with a p53-expressing adenovirus (Cregan *et al.* 2002), or the excitotoxin *N*-methyl-D-aspartate (NMDA) (Yu *et al.* 2002). In a model of neurotrauma, the translocation of AIF in selected brain areas could be correlated with genomic DNA degradation to ~50 kb fragments (which is a hallmark of AIF-mediated nuclear apoptosis) (Zhang *et al.* 2002). We have shown that DNA-damage-induced AIF translocation and apoptosis depends on the presence of p53 and its transcriptional target Bax (Cregan *et al.* 2002). Under excitotoxic conditions we have shown that NMDA-induced release of AIF, is PARP-dependent. Microinjection of a neutralizing antibody recognizing a surface-exposed domain of AIF prevents cell death (Cregan *et al.* 2002; Yu *et al.* 2002) but caspase inhibition alone has no beneficial effect on cell survival (Braun *et al.* 2001; Yu *et al.* 2002; Zhang *et al.* 2002). Assuming that the anti-AIF antibody has no additional effects this suggests that AIF contributes to neurotoxicity. Taken together these data suggest that neurons are susceptible to both caspase dependent and independent death programs and that AIF can participate in both. In NMDA excitotoxicity the death program runs in a serial manner. Blocking NOS, PARP or AIF prevents the downstream events from occurring and prevents death. Caspase activation is a consequence of AIF release and does not contribute to cell death as inhibition of caspases does not produce cell survival. In neurotoxicity due to p53 activation following DNA damage parallel pathways are activated. The caspase pathway is primary but blocking this pathway merely delays cell death. A parallel p53 pathway also activates the AIF



pathway. In order to elicit neuroprotection both parallel death programs, the caspase and AIF pathways, must be blocked. This type of parallel death machinery might be important in non-NMDA excitotoxicity elicited by AMPA or kainate.

Summary In the CNS, injury is mediated by a carefully choreographed series of events initially triggered by calcium influx through the NMDA glutamate receptor activating nNOS and mitochondrial respiration (Figure 3). The production of NO and superoxide anion result in the formation of peroxynitrite, which can diffuse from the mitochondria to damage various cellular constituents. In the nucleus the nicks in DNA induced by peroxynitrite activate the enzyme PARP-1 which consumes NAD in the process of generating PAR and modifying proteins with PAR. Activation of PARP-1 signals to the mitochondria and AIF is released. Translocation of AIF to the nucleus results in large-scale DNA fragmentation and nuclear shrinkage. This is likely the final commitment point to cell death. Genetic deletion studies and pharmacologic inhibition studies indicate that this pathway is critically important in several models of neurologic injury including models of stroke and Parkinson's disease. However, serial pathways may also be activated in some neurologic diseases. It is important to identify all pathways and order the sequence of events to better understand the signal cascades that result in neuronal death. It is the hope that a better understanding of these events will lead to identification of new target molecules to treat patients with neurologic disease.



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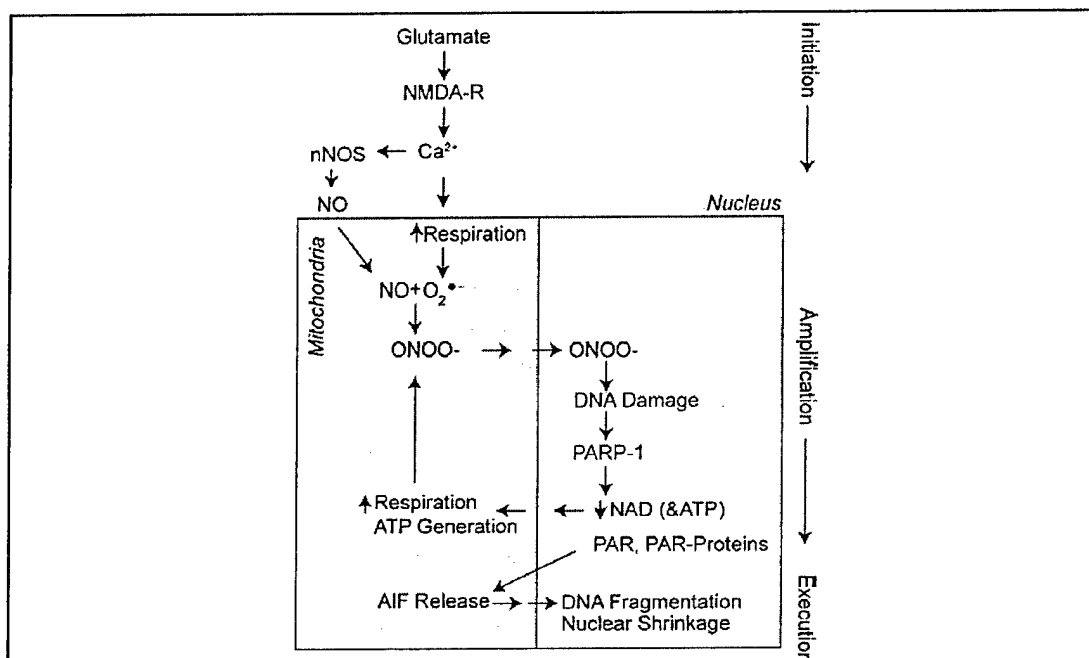


Figure 3. Schematic of the ROS, PARP-1, AIF death program in excitotoxicity.

In the initiation phase of neurotoxicity, activation of NMDA glutamate receptors leads to increased intracellular calcium that activates neuronal nitric oxide synthase (nNOS) producing nitric oxide (NO). Neuronal activation will also result in increased oxidative phosphorylation and subsequently increased superoxide anion production in the mitochondria. Superoxide is not membrane permeable and resides largely in the mitochondria where it is generated. NO and superoxide anion react to form the potent oxidant, peroxynitrite. Peroxynitrite generation can trigger an amplification phase of neurotoxicity by attacking mitochondrial proteins in the electron transport chain including complex I and IV as well as the superoxide scavenging enzyme, manganese superoxide dismutase (MnSOD). This initiates a viscous cycle of peroxynitrite generation through sustained superoxide anion generation. Peroxynitrite is membrane permeable and can move to the nucleus triggering DNA strand breaks. Damaged DNA activates poly (ADP-Ribose) polymerase (PARP) resulting in the synthesis of PAR polymers, ribosylation of proteins and consumption of NAD and ATP. These PARP dependent events signal to the mitochondria to release apoptosis inducing factor (AIF) that translocates to the nucleus. In the nucleus AIF triggers large scale DNA fragmentation and nuclear condensation. These nuclear changes in neurons are likely the final commitment and execution point in the neurotoxic cascade. Subsequent to these events cytochrome c is released and caspases are activated. Blocking these events does not prevent NO/PARP dependent neurotoxicity but may be important in degradation of the cell.



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Neuronal Apoptosis: BH3-only Proteins the Real Killers?

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Abstract

At current there is a poor understanding of the events that lead up to neuronal apoptosis that occurs in neurodegenerative diseases and following acute ischemic episodes. Apoptosis is critical for the elimination of unwanted neurons within the developing nervous system. The Bcl-2 family of proteins contains pro and anti apoptotic proteins that regulate the mitochondrial pathway of apoptosis. There is increasing interest in a subfamily of the Bcl-2 family, the BH3-only proteins and their pro-apoptotic effects within neurons. Recently ischemic and seizure induced neuronal injury has been shown to result in the activation of the BH3-only protein, Bid. This protein is cleaved and the truncated protein (tBid) translocates to the mitochondria. The translocation of tBid to the mitochondria is associated with the activation of outer mitochondrial membrane proteins Bax/Bak and the release of cytochrome-c from the mitochondria. ER stress also has been implicated as a factor for the induction of apoptosis in ischemic neuronal injury. The induction of ER-stress in hippocampal neurons has been shown to activate expression of *bb3/PUMA*, a member of the BH3-only gene family. Activation of PUMA is associated with the activation and clustering of the pro apoptotic Bcl-2 family member Bax and the loss of cytochrome-c from the mitochondria.

Introduction

Excitotoxic neuron death has been implicated in the pathogenesis of ischemic, traumatic and epileptic brain injury (Choi, 1994). Following prolonged glutamate receptor over activation there is extensive necrotic cell death. This is characterized by increased free radical production, a collapse in the mitochondrial membrane potential ($\Delta\Psi_m$), disruption of Ca^{2+} homeostasis, ATP depletion and an increase in cellular volume (Choi, 1987; Tymianski et al., 1993; Ankarcrona et al., 1995; Budd and Nicholls, 1996; White and Reynolds, 1996). However, when glutamate receptor activation is only transient, a more delayed cell death may result (Ankarcrona et al., 1995; Budd et al., 2000; Luetjens et al., 2000; Ward et al., 2000). This delayed cell death is associated with a release of cytochrome-c from the mitochondria and a collapse of the $\Delta\Psi_m$ (Budd et al., 2000; Lankiewicz et al., 2000; Luetjens et al., 2000; Ward et al., 2000). The molecular mechanism of the mitochondrial cytochrome-c release during excitotoxic neuronal cell death remains unresolved.

In the "classical" mitochondrial apoptosis signalling pathway, the release of cytochrome-c requires the pro-apoptotic Bcl-2 family members Bax or Bak (Wei et al., 2001). These proteins are believed to form pores that make the outer mitochondrial membrane sufficiently permeable for the release of inter-membrane proteins that include cytochrome-c (Kuwana et al., 2002). For this to occur, Bax and Bak must undergo conformational changes and insert into the outer mitochondrial membrane (Goping et al., 1998; Eskes et al., 1998). The transcriptional induction or the post-translational activation of Bcl-2-homolgy domain-3 (BH3)-only proteins is required to trigger the activation of Bax and Bak (Huang and Strasser, 2000), which can be antagonized by the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL.

BH3-Only Proteins

The BH-3-only proteins are a subfamily of the Bcl-2 protein family that are essential initiators of programmed cell death through the activation of Bax and Bak. All the members of this protein



family contain a short amino acid (9-16 amino acids) BH3- domain, however they don't possess a very strong structural homology (Huang and Strasser 2000). The BH3 domain is essential for the binding of these BH3-only proteins to both pro- and anti-apoptotic members of the Bcl-2 family. In *C. elegans* a single BH3-only protein, EGL-1, is required for the initiation of programmed cell death (Conradt and Horvitz, 1998), however, in mammals there are at least 12 BH3-only proteins (See Table 1). The diversity of these BH3-only proteins is reflected in the different modes of activation. They operate by either blocking the actions of the anti-apoptotic proteins (Bcl-2 Bcl-xL) therefore facilitating apoptosis or to promote apoptosis through the activation of pro apoptotic proteins (Bax and Bak).

BH3-only Proteins	Stimulus, Upstream regulators	Mode of activation	Reference
Bad	Growth factor withdrawal, Seizure induced cell death	Phosphorylation	Zha et al 1996 Henshall et al., 2001
Bid	Ischemia, Seizure induced cell death	Lipid modification Proteolytic cleavage	Desagher et al., 1999, Wei et al., 2001 Plesnila et al., 2001
Bik		Phosphorylation	Biswas and Greene 2002
Bim	Growth factor withdrawal, FKHRL1 JNK	Transcriptional induction Phosphorylation Translocation	Puthalakath et al., 1999 Putcha et al., 2001
Blk		Phosphorylation	Hedge et al 1998
Bmf	anoikis (=matrix detachment)	Phosphorylation Translocation	Puthalakath et al., 2001
BNIP3	Hypoxia	Transcriptional induction	Yasuda et al 1998
NIX	Hypoxia	Transcriptional induction	Yasuda et al 1998
Hrk	JNK	Transcriptional induction	Inohara et al., 1998
PUMA	p53 ER stress	Transcriptional induction	Yu et al 2001
Noxa	p53, Hypoxia	Transcriptional induction	Oda et al 2000 Kim et al. 2004
Spike		Transcriptional induction	Mund et al 2003

Table 1. Mammalian BH3-only proteins. Stimuli and regulation



Excitotoxic Injury

In recent years it is becoming more evident that BH3-only proteins are integrally involved with the apoptotic neuronal death cascade. Neurons from mice deficient of the BH3-only protein Bid have been shown to be resistance to ischemic injury in vivo, as well as hypoxic and excitotoxic injury in vitro (Plesnila et al., 2001). The activation of Bid and the subsequent cleavage of Bid to truncated form (tBid) is an essential component of most forms of death receptor-mediated apoptosis (Li et al., 1998; Luo et al., 1998). During an ischemic episode or seizure induced neuronal death, Bid is truncated to its 15kDa form that targets the mitochondria (Plesnila et al., 2001; Henshall et al., 2001). This cleavage of bid has been shown to occur through caspase-8 (Li et al., 1998; Luo et al., 1998). tBid is then translocated to the mitochondria where it activates Bax or Bak and induces the release of cytochrome-c from the mitochondria (Eskes et al., 2000; Wei et al., 2000). As caspase activation may only be very marginal in excitotoxic neuronal apoptosis (Armstrong et al., 1997; Budd et al., 2000; Lankiewicz et al., 2000) this suggests that the activation of Bid may trigger excitotoxic neuronal injury through another pathway (Chen et al., 2001; Stoka et al., 2001). There is also increasing evidence that Bim may play a significant role in the regulation of neuronal vulnerability in seizure induced neuronal injury (Shinoda et al., in press)

ER Stress

It has been suggested that ER stress may also contribute to the induction of neuronal apoptosis injury following ischemia (Paschen and Frandsen, 2001). ER stress is the term given to any condition that results in the accumulation of unfolded or misfolded proteins within the ER lumen (Kauffman, 1999). In rodent cells, ER stress-induced cell death has been shown to involve the activation of ER-resident caspase-12, which subsequently activates executioner caspases such as caspase-3 (Nakagawa and Yuan 2000, Nakagawa et al., 2000). However, there is increasing evidence that ER stress may activate the mitochondrial apoptotic pathway that results in the release of cytochrome-c from the mitochondria (Häcki et al., 2000., Annis et al., 2001; Wei et al 2001). This requires an increase in the permeability of the outer mitochondrial membrane that is triggered by Bax and Bak (Desagher and Martinou, 2000; Wei et al., 2001). In a recent study Reimertz et al (2003) used tunicamycin to induce ER stress in hippocampal neurons. Tunicamycin prevents protein glycosylation and results in the build up of malformed proteins within the lumen of the ER. This in turn results in the characteristic unfolded protein response (UPR). Following tunicamycin there is an increase in the expression of a number of different genes that are typically involved in the UPR response, such as *BIP* and *GRP 94* (for review see Kaufman et al 1999). These molecular chaperones help relieve ER stress by promoting protein folding and keeping proteins in a folding competent state. Following ER stress induced by tunicamycin, there is induction of the *bbc3/PUMA* protein and subsequent activation of the mitochondrial apoptosis pathway indicated by the release of cytochrome-c from the mitochondria (Reimertz et al., 2003). In addition to ER stress, *Bbc3/PUMA* is also activated after transient forebrain ischemia. Furthermore, overexpression of *Bbc3/PUMA* is sufficient to trigger apoptosis in neuronal cells and cells deficient in *bbc3/PUMA* showed dramatically reduced apoptosis in response to ER stress (Reimertz et al., 2003).

It is apparent that the BH3-only proteins play an integral part in the apoptotic pathways that are associated with ischemic and seizure induced neuronal injury. Through a more detailed understanding of these pathways we will gain a new insight into the regulatory pathways that control neuronal injury and through this we may find the new targets for future drug development.

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Protective Roles of CNS Mitochondria.

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With much focus upon the role of mitochondria in apoptotic signaling and the permeability transition in acute necrotic injury, the roles mitochondria play in generating energy, maintaining homeostasis and protecting cells from injury may appear as secondary or lesser functions. However, the role of mitochondria in preserving cell health is clearly the more dominant function. First and foremost is the principal role of mitochondria in generating ATP. Concomitant with generating ATP, reducing molecular oxygen through the electron transport chain prevents its spurious involvement in oxidation of other important cellular constituents; proteins, lipids, or oligonucleotides (Skulachev, 1996). Electron transport and oxidative phosphorylation harness the high redox potential of O₂, transforming it into the high energy phosphate bond of ATP. With O₂ as substrate, its flux becomes part of a highly regulated set of reactions, and thus it is less likely to participate in spurious, harmful oxidative activity. Residual reactive oxygen species are largely controlled either by transmutase reactions or by reducing their production by increasing O₂ utilization through the electron transport chain. Mild uncoupling, or even induction of the permeability transition, could serve to temporarily depolarize mitochondria and decrease production of reactive oxygen species. Thus, mitochondria benefit the cell by both producing energy efficiently and by largely detoxifying oxygen. (Skulachev, 1996).

Mitochondria also function to maintain cellular homeostasis by sequestering calcium (Nicholls, 1985). Mitochondrial membrane potential is a potent driving force for moving Ca²⁺ up a concentration gradient into the mitochondrial matrix. Once inside, free Ca²⁺ is controlled by its solubility in association with phosphate, permitting temporary storage of Ca²⁺ in a relatively inactive form (Chalmers and Nicholls, 2003). Mitochondria act as a transient Ca²⁺ sink during rapid events that increase cytosolic Ca²⁺, such as the neuronal action potential (Werth and Thayer, 1994), and during longer lasting events associated with Ca²⁺ waves (Landgraf et al., 2004). Under stressful conditions, when neurons are challenged by overexposure to the excitotoxic neurotransmitter glutamate, mitochondrial sequestering of Ca²⁺ may figure critically into the ability of the neuron to prevent necrosis. Several recent studies of the often destructive mitochondrial permeability transition have revealed new ways in which mitochondria may actually be acting to preserve mitochondrial and cellular function.

In isolated mitochondrial preparations, bolus addition of a substantial Ca²⁺ load or repeated additions of smaller Ca²⁺ doses eventually leads to mitochondrial depolarization, swelling and an eventual inability of mitochondria to sequester the calcium. Evidence for the occurrence of this permeability transition in situ has been gathered from astrocytes, neurons and a plethora of non-neuronal cells (Dubinsky and Levi, 1998; Kristal and Dubinsky, 1997; White and Reynolds, 1996; Jordan et al., 2003). When neuronal cytosolic Ca²⁺ is elevated with an excitotoxic dose of glutamate, mitochondria depolarize in a manner that may or may not be sensitive to inhibitors of the permeability transition (Brustovetsky and Dubinsky, 2000b; Budd et al., 2000; Reynolds, 1999).



Ambiguity surrounding a role of the permeability transition in excitotoxicity may lie in the variety of responses receiving that name and in the differential susceptibility of mitochondria from different neuronal populations. The conductance pathways opened by a mPT may vary. In single channel recordings, large conductance mitochondrial channels may be activated spontaneously or by peptides, Bcl-xL, or Ca^{2+} (Muro et al., 2003; Kushnareva et al., 2001; Jonas et al., 2003). Any or all of these could contribute to events ascribed to the permeability transition. In isolated mitochondrial preparations, Ca^{2+} may trigger a high conductance response, associated with simultaneous depolarization and swelling (Brustovetsky and Dubinsky, 2000a; Brustovetsky and Dubinsky, 2000a). While mitochondria may recover from such an event, it often results in mitochondrial disintegration and may be part of a regulated autophagic pathway (Lemasters et al., 2002). Ca^{2+} may also activate a limited permeability that only depolarizes mitochondria without causing swelling (Brustovetsky and Dubinsky, 2000a; Brustovetsky and Dubinsky, 2000a). Initiated under conditions of limited substrate availability, isolated CNS mitochondria respond to external Ca^{2+} with such a sustained depolarization. This depolarization drastically reduces the driving force for Ca^{2+} influx, limiting the mitochondria's ability to sequester Ca^{2+} (Brustovetsky and Dubinsky, 2000a; Brustovetsky and Dubinsky, 2000a). Such a response should protect mitochondria against more severe damage. Indirectly it protects the neuron from the eventuality of a compromised metabolism, preserving mitochondrial integrity for future ATP generation. Seen from the mitochondria's point of view, this limited permeability pathway, which may be a component of or precursor to the high conductance mPT, is an attempt to prevent the latter from occurring. Preventing Ca^{2+} entry precludes accumulation of matrix free Ca^{2+} to levels sufficient for induction of a high conductance mPT. Among the heterogeneous responses of cultured hippocampal neurons, mitochondrial depolarization accompanying glutamate-induced sustained increases in intracellular calcium may reflect opening of such low conductance pathways (Fig. 1). In such cases, FCCP addition to fully depolarize mitochondria and unload accumulated calcium results in no further increase in cytosolic Ca^{2+} (Brustovetsky and Dubinsky, 2000a; Brustovetsky and Dubinsky, 2000a). In a similar manner, chemically-induced transient

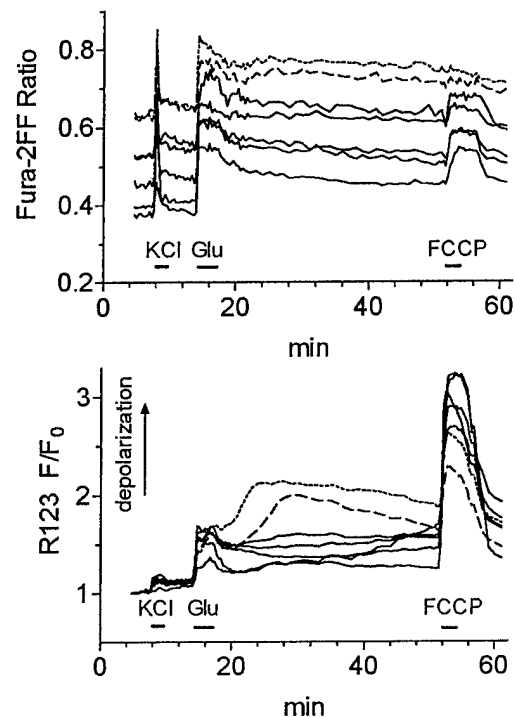


Fig. 1. Cultured hippocampal neurons loaded with the low affinity Ca^{2+} dye, fura-2FF and the mitochondrial membrane potential sensitive dye, R123, display mixed reactions to $500\mu\text{M}$ glutamate. Neurons with the most depolarized mitochondria display the highest sustained calcium levels. Mitochondrial depolarization with FCCP reveals an absence of mitochondrially stored calcium. Reprinted with permission from J. Neurosci. 20:103.



mitochondrial depolarization of cultured cortical neurons is neuroprotective (Stout et al., 1998). In cerebellar cultures, similar mitochondrial depolarization can prevent glutamate-induced delayed calcium deregulation, a precursor to cell death (Nicholls and Budd, 1998). Admittedly, while depolarization via activation of a low conductance permeability may be protective of the mitochondria, it may not always result in endogenous neuroprotection. Elevated cytosolic Ca^{2+} can initiate a variety of other deleterious pathways that result in cell death. However, preserving mitochondrial integrity may result in these other pathways leading to a slower and more tidy apoptotic process, fueled by mitochondrial generated ATP, rather than an acute necrotic demise.

Variability in regional brain mitochondria may also contribute to the controversy regarding whether or not the mPT participates in excitotoxicity. Strong arguments have been made against its participation in glutamate-induced delayed calcium deregulation in cultured cerebellar granule cells (Nicholls and Budd, 1998). However isolated cerebellar mitochondria are less sensitive to Ca^{2+} induced swelling than mitochondria from cortex or hippocampus (Friberg et al., 1999).

Hippocampal neurons variably display morphological changes in mitochondria after excitotoxic glutamate exposure (Fig. 2) (Dubinsky and Levi, 1998), consistent with the high sensitivity of hippocampal mitochondria to Ca^{2+} -induced swelling (Friberg et al., 1999). Similarly, isolated rat striatal mitochondria appear more susceptible to Ca^{2+}

induced depolarization and swelling than cortical mitochondria (Brustovetsky et al., 2003). Such differential sensitivity may contribute to the initially greater susceptibility of striatal neurons in degenerative diseases such as Huntington's Disease. To address this question, we examined regional brain mitochondria from a very slowly progressing mouse model of HD in which long polyglutamine expansions have been placed in exon 1 of the mouse Huntington gene (Wheeler et al., 2000). Indeed when striatal and cortical mitochondria from Q111 mutant huntingtin knock-in mice were examined from various ages, the initially more susceptible striatal mitochondria changed with increasing age in a polyglutamine dependent manner (Fig. 3). Striatal mitochondrial Ca^{2+} sensitivity decreased until these mitochondria became equally sensitive to the cortical mitochondria. The shift in

Ca^{2+} sensitivity occurred very early in the disease progression, at the time that nuclear localization of mutant huntingtin first occurred (Wheeler et al., 2000). Thus in the early stages of disease progression, the striatal mitochondria became more resistant to induction of the permeability transition. This compensatory response again demonstrates a mitochondrial propensity for self preservation. A higher Ca^{2+} threshold will make neurons more tolerant of increases in intracellular Ca^{2+} . However, this adaptive strategy may only work in the short term. If uncoupling and the permeability transition are indeed safety valves or ways for cells to detoxify extra oxygen by maximally running electron transport (Skulachev, 1996), then by raising the threshold for onset of the permeability transition, neurons may increase their risk for cumulative oxidative damage. As with any stressed tissue that mounts a compensatory response, overall susceptibility of the entire cell to other degenerative processes may be increased.

Similar adaptive mitochondrial behavior has been previously reported in precancerous liver cells of rats fed the carcinogen 2-acetylaminofluorene (Klohn et al., 2003). Liver mitochondria harvested from animals on this diet for 3-4 weeks had a greatly decreased sensitivity to Ca^{2+} activation of the mPT. While the epigenetic process leading to this increased resistance remains

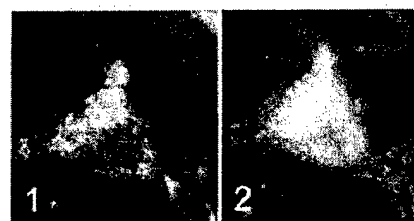


Fig. 2. Changes in mitochondrial morphology often associated with excitotoxicity. Cultured hippocampal neuron stained with mitotracker green (1) initially and (2) 20 min after 5 min of 500 μM glutamate.



unknown, the mitochondrial response is clearly one of self-preservation, both for the mitochondria and for the immediate livelihood of the hepatocytes. Their eventual progression into neoplastic cells, resistant to cell death, may be a logical extension of the initially protective response.

Thus, in addition to their primary roles in energy generation, oxygen fixation and calcium sequestration, mitochondria may adapt to locally stressful conditions with subtle protective behaviors. Depolarization resulting from uncoupling or Ca^{2+} -activated pathways may prevent mitochondrial disintegration and relieve the cell of excess oxygen (Skulachev, 1996). Shifting a cell's sensitivity to Ca^{2+} induction of a high conductance permeability transition may avoid acute necrosis, allowing cellular homeostasis to adapt to stressful conditions. Mitochondrial self-protection may be an early initial response to the stresses of elevated cytosolic calcium. Such adaptive behaviors may benefit the mitochondria themselves, their host cells and the whole tissue.

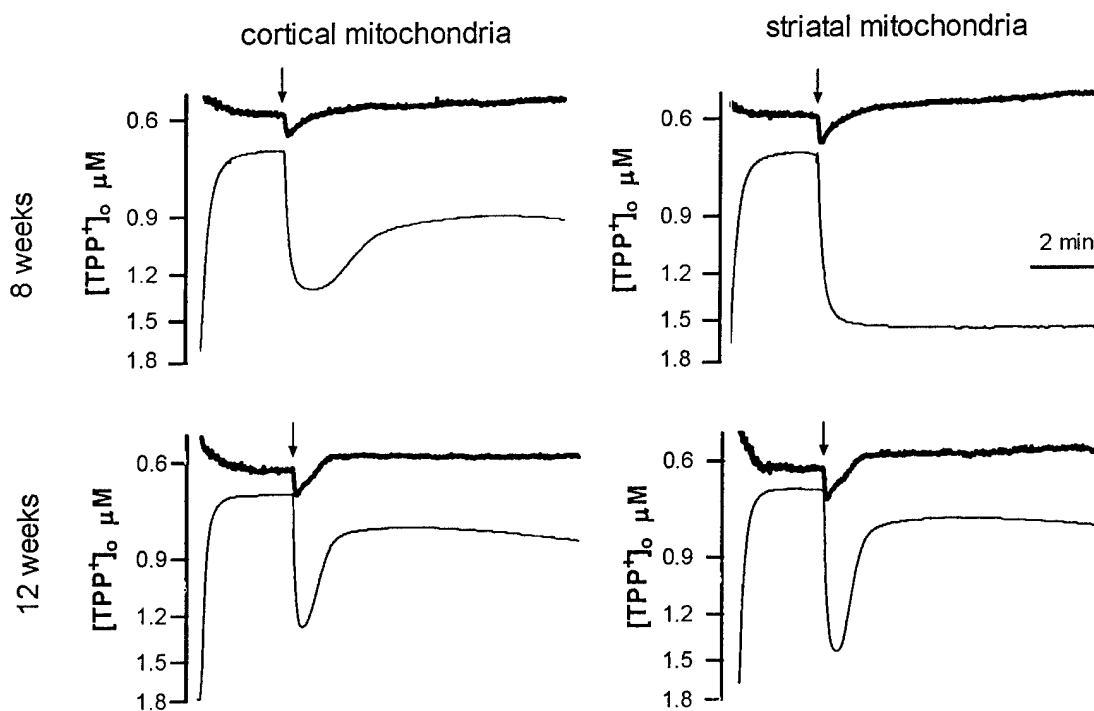


Fig. 3. Isolated cortical and striatal mitochondria from Q111 mice 8 and 12 weeks of age. Mitochondrial membrane potential was measured by the uptake of TPP^+ (thin lines) and swelling was monitored by light scattering (thick lines) (Brustovetsky et al., 2003). $0.3 \mu\text{mol Ca}^{2+}$ per mg mitochondrial protein was added at the arrows. In older Q111 mice, striatal mitochondria retain the same Ca^{2+} sensitivity as observed at 12 weeks.

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Ammonia Neurotoxicity and the Mitochondrial Permeability Transition

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Introduction

Ammonia is a neurotoxin that has been strongly implicated in the pathogenesis of hepatic encephalopathy (HE), an important cause of morbidity and mortality in patients with severe liver failure. It is also an important factor in inborn errors of the urea cycle, Reye's syndrome, organic acidurias, valproate toxicity, transient hyperammonemia in infants, and idiopathic hyperammonemia.

The pathology of hyperammonemia, particularly HE, suggests that astrocytes play a crucial role in this condition (Norenberg, 1987). Astrocyte swelling represents the principal component of acute HE, while the presence of Alzheimer type II astrocytes is the main histological finding in chronic HE. No significant or consistent neuronal changes have been identified (Norenberg, 1981). Because of the critical role of astrocytes in neurotransmission and CNS bioenergetics, we have proposed that astroglial dysfunction (gliopathy) and associated derangement in glial-neuronal interactions represent major aspects in the pathogenesis of ammonia neurotoxicity (Norenberg, 1997).

Cerebral ammonia is chiefly metabolized to glutamine in astrocytes, due to predominant localization of glutamine synthetase in these cells (Norenberg and Martinez-Hernandez, 1979). Physiological levels of glutamine thus formed in astrocytes is released into the extracellular space and is taken up by neurons to generate glutamate and ammonia, a reaction mediated by phosphate-activated glutaminase (PAG). In addition, glutamine can also be metabolized to glutamate and ammonia in astrocytes, as evidenced by studies on astrocytes in culture (Kvamme *et al.*, 1992) as well as in vivo (Subbalakshmi and Murthy, 1985) possess PAG.

This article highlights the role of the mitochondrial permeability transition (MPT) as a major factor in the cellular dysfunction associated with ammonia neurotoxicity. The role of oxidative stress will be emphasized as a causal factor in the induction of the MPT. Mitochondrial dysfunction resulting from ammonia neurotoxicity as a consequence of the MPT will be discussed. Lastly, recent concepts on potential mechanism(s) of the ammonia-induced MPT will be presented.

CEREBRAL ENERGY METABOLIC FAILURE IN AMMONIA TOXICITY

The concept that ammonia disturbs cerebral energy metabolism has long been proposed (see Rama Rao and Norenberg, 2001 and references therein). Ammonia is known to interfere with various metabolic pathways of cerebral energy metabolism including inhibition of α -ketoglutarate dehydrogenase; stimulate Na^+, K^+ -ATPase resulting in depletion of ATP; impair the oxidation of pyruvate and glutamate; disturb the operation of the malate-aspartate shuttle; reduce state III mitochondrial respiration; and inhibit the activity and expression of electron transport chain enzymes. Some of the abnormalities have been reproduced in cultured astrocytes exposed to pathophysiological concentrations of ammonia. In addition, several studies have shown morphologic changes in mitochondria in HE/hyperammonemia, principally swelling of the matrix and intracristal space (Norenberg, 1977; Gregorios *et al.*, 1985b; Norenberg *et al.*, 2002).

OXIDATIVE STRESS IN AMMONIA TOXICITY

Oxidative stress is an evolving concept in HE and ammonia toxicity. Increased superoxide production and reduced activities of antioxidant enzymes have been reported in brains of rats subjected to acute ammonia toxicity (Kosenko *et al.*, 1997). Consistent with these findings, biphasic responses of total glutathione (GSH) were identified in cultured astrocytes exposed to 5 mM NH_4Cl .



At early time points (up to 6 h) GSH levels were reduced by ammonia, whereas at later time points (up to 72 h), a progressive increase in GSH content occurred (Murthy *et al.*, 2000). Lowered levels of GSH in astrocytes in early phase of ammonia exposure is consistent with the concept that ammonia induces oxidative stress in astrocytes. The later increase in GSH may represent an adaptive response to oxidative stress.

To examine the cellular basis of oxidative stress in ammonia toxicity, free radical production was measured employing the fluorescent probe 5-(and-6)carboxy-2'-7'-dichlorofluorescein diacetate (DCFDA). These studies demonstrated that ammonia stimulated the production of free radicals in a dose-dependent manner. These data also disclosed that ROS levels remained elevated for at least 4h after exposure to ammonia. At the earliest time point (3 min) there was a robust increase in free radical production followed by a transient but significant reduction up to 2h (but still higher than control); at 4 h the increase was similar to that observed at the 3 min time point (Murthy *et al.*, 2001; Rama Rao *et al.*, 2003). This pattern of increase in ROS production by ammonia (2-4 h) is consistent with a concomitant decrease (up to 6h) in astrocytic GSH levels as described above.

THE MITOCHONDRIAL PERMEABILITY TRANSITION

The potential involvement of mitochondrial dysfunction and oxidative stress in ammonia neurotoxicity prompted our investigation into the possible role of the mitochondrial permeability transition (MPT) in hyperammonemia. The MPT is characterized by a sudden increase in the permeability of the inner mitochondrial membrane to small molecules (<1500 Da). This is due to the opening of a specific permeability transition pore in the inner mitochondrial membrane, usually in response to an increase in mitochondrial Ca^{2+} levels. This leads to a collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$) that is created by the pumping out of protons by the electron transport chain. Loss of the $\Delta\Psi_m$ leads to colloid osmotic swelling of the mitochondrial matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis, and the generation of ROS. For reviews, see Zoratti and Szabo (1995) and Bernardi *et al.* (1998). The most specific blocker of the MPT is cyclosporin A (CsA), which competitively inhibits the mitochondrial matrix protein cyclophilin D from binding to pore domains (Crompton *et al.*, 1998).

To determine whether ammonia treatment of cultured astrocytes was associated with a change in the $\Delta\Psi_m$, a consequence of the MPT, astrocytes were treated with 5 mM NH_4Cl and examined for changes in the $\Delta\Psi_m$ using the potentiometric fluorescent dyes JC-1 and TMRE. Astrocytes exposed to ammonia showed a significant dissipation of the $\Delta\Psi_m$ in a time- and concentration-dependent manner. These studies also demonstrated that pretreatment with CsA (1-5 μM) protected the ammonia-induced dissipation of the $\Delta\Psi_m$ (Bai *et al.*, 2001; Rama Rao *et al.*, 2003) (Figure 1), suggesting that ammonia was inducing the MPT.

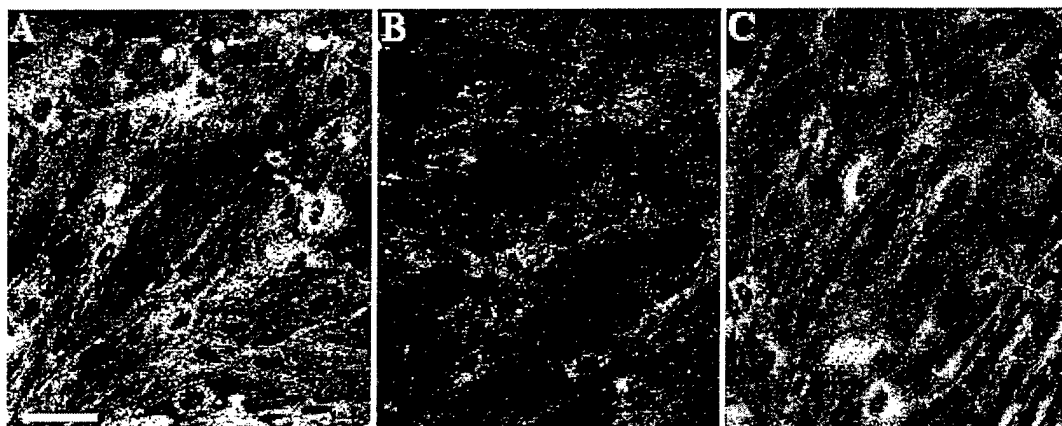


Figure 1: Effect of 5 mM NH_4Cl on TMRE fluorescence in cultured astrocytes. Cells were loaded with 25 nM TMRE for 20 min. **A.** Control astrocytes show prominent fluorescence. **B.** Ammonia-treated astrocytes show decreased fluorescence. **C.** Astrocytes treated with 1 μM CsA and ammonia is similar to control. Scale bar, 10 μm

To directly visualize permeability changes in mitochondria *in situ*, the calcein fluorescence method was employed (Petronilli *et al.*, 1999). Calcein/AM enters cells and becomes fluorescent upon de-esterification. Co-loading of cells with cobalt chloride quenches the fluorescence in the cell, except in mitochondria, since cobalt is impermeable across mitochondrial membranes. However, during induction of the MPT, cobalt enters mitochondria and quenches the calcein fluorescence. Treatment of cultured astrocytes with ammonia (24 h) caused a significant reduction in the fluorescent intensity of calcein, which was significantly blocked by pretreatment with CsA (5 μM) (Figure 2).

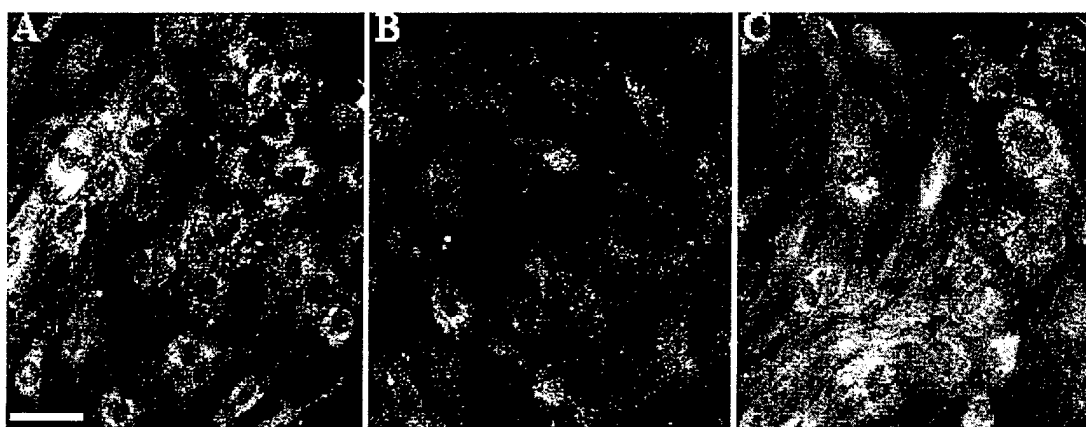


Figure 2: Induction of the MPT in astrocytes by ammonia as demonstrated by calcein fluorescence. **A.** Control astrocytes loaded with 1 μM calcein and quenched with cobalt show brightly stained mitochondria. **B.** Astrocytes treated with ammonia (5 mM) for 24 h and then loaded with calcein show a significant loss of mitochondrial calcein fluorescence, consistent with the induction of the MPT. **C.** Co-treatment with CsA (1 μM) prevents the loss of calcein fluorescence by ammonia. Scale bar, 10 μm .



The ammonia-induced MPT in cultured astrocytes was significantly attenuated by various antioxidants, including SOD (25 U/ml), catalase (250 U/ml), desferroxamine (40 μ M, N-*t*-butyl- α -phenyl-nitrone (PBN; 250 μ M), supporting the notion that oxidative stress plays a major role in the ammonia-induced MPT in astrocytes (Jayakumar *et al.*, 2002).

ROLE OF GLUTAMINE IN THE MECHANISM OF AMMONIA NEUROTOXICITY

While ammonia is believed to be responsible for the neurological abnormalities associated with HE and other hyperammonemic syndromes, growing evidence supports the view that glutamine, a byproduct of ammonia metabolism, plays a major role in the deleterious effects of ammonia. Various abnormalities associated with ammonia toxicity such as seizures, depressed glucose utilization, altered CNS metabolism, vascular CO₂ responsiveness, edema, and astrocyte swelling can be blocked by administration of methionine sulfoxamine (MSO), an inhibitor of glutamine synthetase (Rama Rao *et al.*, 2003b and references therein).

Earlier studies showed that MSO completely blocked the effect of ammonia on the MPT (Bai *et al.*, 2001), as well as free radical production (Murthy *et al.*, 2001). These findings suggested that glutamine was mediating the effects of ammonia on the MPT and free radical formation. Subsequent studies have examined the role of glutamine directly. Cultured astrocytes treated with glutamine (4.5-7 mM for 24 h) caused a significant dissipation of $\Delta\Psi_m$ as well as decreased mitochondrial calcein fluorescence, both of which were completely blocked by CsA (Rama Rao *et al.*, 2003). In addition, glutamine significantly increased free radical production in cultured astrocytes, which was also completely blocked by CsA (Jayakumar *et al.*, 2004).

To investigate the potential mechanism by which glutamine induces free radicals and the MPT, cultured astrocytes were treated with 6-diazo-5-oxo-L-norleucine (DON; 1 mM), an inhibitor of phosphate-activated glutaminase (PAG). DON completely blocked the glutamine-induced free radical production (Jayakumar *et al.*, 2004). Since essentially all of the glutamine is metabolized in mitochondria by PAG, high levels of ammonia will be generated in these organelles leading to the production of free radicals and the induction of the MPT. We envision glutamine acting as "Trojan horse" by providing high levels of ammonia, leading to oxidative stress and mitochondrial dysfunction.

ASTROCYTIC MITOCHONDRIA ARE MORE VULNERABLE TO THE AMMONIA-INDUCED MPT

It is noteworthy that astrocytic rather than neuronal mitochondria are predominantly affected by ammonia (Bai *et al.*, 2001). Similarly, glutamine had no effect on free radical production in cultured neurons (Jayakumar *et al.*, 2004). There are two possibilities to explain these findings. First, there is evidence of heterogeneity of mitochondria among neurons and astrocytes (Blokhuys and Veldstra, 1970), and it is possible that neuronal mitochondria may be more resistant to induction of the MPT by ammonia. Supporting this possibility, Fiskum *et al.*, (2000) demonstrated a greater resistance of neuronal mitochondria to the effects of Ca²⁺ overload and the subsequent induction of the MPT as compared with astrocytic mitochondria. Second, the selective vulnerability of astrocytes to ammonia-induced MPT may be due to high levels of glutamine in astrocytes since ammonia is metabolized to glutamine in astrocytes but not in neurons.

ROLE OF THE MPT IN ASTROCYTE SWELLING

Astrocyte swelling represents a significant component of the brain edema in fulminant hepatic failure (FHF) (Córdoba and Blei, 1996). While the mechanism of edema associated with FHF is not



completely understood, elevated ammonia levels have been strongly implicated in this disorder (Clemmesen *et al.*, 1999). Studies employing cultured astrocytes (Norenberg *et al.*, 1991) and brain slices (Ganz *et al.*, 1989) exposed to pathophysiological concentrations of ammonia have demonstrated prominent astrocyte swelling. More recently, ammonia has been shown to upregulate the water channel protein aquaporin4 (AQP4), suggesting that AQP4 may be responsible for astrocyte swelling (Rama Rao and Norenberg, 2003a). Collectively, there is compelling evidence that supports a major role of ammonia in the astrocyte swelling associated with hyperammonemia.

Since ammonia has been shown to induce the MPT and mitochondrial dysfunction, the role of the MPT on astrocyte swelling was assessed. Pretreatment of cultured astrocytes with different concentrations of CsA (0.1-1 μ M) significantly blocked the astrocyte swelling caused by ammonia. Parallel studies also demonstrated that CsA treatment significantly blocked the ammonia-mediated increase in AQP4 expression (Rama Rao and Norenberg, 2003b). Additionally, antioxidants significantly blocked the ammonia-induced astrocyte swelling (Murthy *et al.*, 2000). These studies support the role of the MPT and oxidative stress in the astrocyte swelling and brain edema associated with hyperammonemic states.

CONCLUDING REMARKS

In summary, ammonia induces the MPT in cultured astrocytes but not in cultured neurons, highlighting the critical role that astrocytes play in the toxic effects of ammonia. These effects of ammonia on the MPT were prevented by cyclosporin A. Ammonia-induced astrocyte swelling was blocked by CsA suggesting a major role of the MPT in this process. Our studies also suggest that glutamine likely mediates the effect of ammonia in the induction of oxidative stress as well as the MPT. We propose that oxidative stress and the MPT represent key pathogenetic factors in ammonia neurotoxicity. These findings provide potential therapeutic targets for HE and other hyperammonemic states.

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The mitochondrial permeability transition as a target for neuroprotection

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Recognition that mitochondria could release triggers of cell death, and that at least one of these triggers, cytochrome c, was sufficient for the latter stages of cell death, highlighted the potential gatekeeper role of mitochondria in cell death cascades. We focus our work in this area on the mitochondrial permeability transition (mPT), which has been defined primarily based on studies in purified liver and heart mitochondria. The term mPT refers to the opening of pores in the inner mitochondrial membrane that allows free diffusion of all solutes <1.5 kD. Therefore, mPT induction leads to loss of the proton gradient, to inability to conduct oxidative phosphorylation, and to a potentially lethal efflux of mitochondrially sequestered calcium into the cytosol. mPT-like events have been observed in mitochondria isolated from CNS tissues (Kristal and Dubinsky, 1997), and mPT induction also has been experimentally associated with release of cytochrome c, AIF, and SMAC/DIABLO, which are the direct activators of the downstream cascades in both caspase-dependent and -independent cell death. Induction of an mPT has been linked to cytotoxicity following pathological insults, including stroke and excitotoxicity (Friberg and Wieloch, 2002). This said, the involvement of mPT in mitochondria from the CNS remains controversial, and available data suggest both PT-dependent and PT-independent events may be initiated by different pathogenic insults. Consistent with this, data from studies at the level of isolated mitochondria, cells, and intact animal models support the existence of "PT-like" events in the nervous system, but equally compelling data, suggest that "PT-like" events in the nervous system must be significantly different than those in the liver or heart, and that cell death in the CNS may often be "PT-independent" (Kristal and Dubinsky, 1997; Berman and Hastings, 1999; DR Andreyev et al, 1998; Andreyev and Fiskum, 1999; Martinou and Green, 2001; Berman et al, 2000). Here, we will focus on some of the pieces of evidence from our work that supports a role for PT in acute neurological injury and in chronic neurodegeneration.

Beyond Calcium and Non-specific ROS – What modifies mPT susceptibility?

One question about the relevance of mPT for cell death *in vivo* has followed from acknowledgement that the conditions used to study mPT *in vitro*, such as the lack of adenine nucleotides, may not translate well to *in vivo* circumstances. A second question concerns whether there may be disease-specific modifiers of mPT induction. We have been trying to address these issues by examining small molecules that may contribute to mPT induction in specific circumstances. Potential small molecule modulators of the mPT include, among others, Zn²⁺, ganglioside GD3 (Kristal and Brown, 1999), the reactive aldehydes 4-hydroxyhexenal (HHE, a lipid peroxidation byproduct, Kristal et al, 1996), and 3,4-dihydroxyphenylacetaldehyde (DOPAL, Kristal et al, 2001), peroxynitrite (Packer and Murphy, 1995), and arachidonic acid (Scorrano et al, 2001). We have also examined whether changes in physiological status (eg, Huntington's disease, impaired respiration) may be associated with changes in susceptibility to mPT induction.

Elevation of free intracellular Zn²⁺ has been observed in both heart and brain after ischemia/reperfusion or excitotoxic insults. Koh et al. found a one-to-one correspondence between neurons with elevated Zn²⁺ and markers of cell death following ischemia-reperfusion (Koh et al.,



1996). Strikingly, in this study, neurons were protected from ischemia-reperfusion injury by chelators of Zn^{2+} but not Ca^{2+} , implicating Zn^{2+} in cell death. These and other studies implicate Zn^{2+} at the cellular and organism level, studies by ourselves and others implicate Zn^{2+} at the mitochondrial level as an mPT inducer and as an inhibitor of α -ketoglutarate dehydrogenase (Brown et al., 2000). Induction of an mPT in the presence of Zn^{2+} shows both quantitative and qualitative similarities and differences relative to that involving Ca^{2+} . As two examples, effects of Zn^{2+} rapidly plateau and reverse, whereas those of Ca^{2+} do not. Zn^{2+} is also associated with a rapid, irreversible depolarization.

Monoamine-derived aldehydes have been suggested as possible toxicants in Parkinson's and Alzheimer's diseases. Consistent with a possible involvement of mPT, we had previously shown that hydroxyhexenal (HHE), a cytotoxic lipid peroxidation byproduct, accelerates mPT induction at femtomolar concentrations (Kristal et al. 1996). We have demonstrated that 3,4-dihydroxyphenylacetaldehyde (DOPAL), the direct MAO metabolite of dopamine, is more cytotoxic in neuronally differentiated PC12 cells than dopamine and several of its metabolites. DOPAL is also a potent mPT inducer (Kristal et al, 2001). These data and others are consistent with a model in which DOPAL-induced mitochondrial damage, including induction of the mPT, contributes to disease progression in PD. Furthermore, DOPEGAL [3,4-dihydroxyphenylglycolaldehyde], the monoamine metabolite of epinephrine and norepinephrine, and a molecule suggested to contribute to Alzheimer's disease, is also a co-stimulators of the mPT.

Huntington's disease is a chronic progressive neurological disease that may also include systemic manifestations, including wasting. These aspects of the disease are modeled in several available transgenic mouse lines, including the R6/2 mouse. We have shown that the advanced stages of illness in this animal model are associated with an increased susceptibility to induction of the mPT in isolated liver mitochondria. This data has both similarities and differences from that previously presented by others (Panov et al, 2002). Increased susceptibility was robustly observed under several different experimental conditions. Comparison with previous work on the mPT in diabetic rodents suggests that the effects observed are not a consequence of the diabetes that occurs in the R6/2 model. Increased susceptibility to mPT induction were independent of alterations in mitochondrial Ca^{2+} transport, endogenous Ca^{2+} load, respiration, or initial mitochondrial membrane potential ($\Delta\Psi$). Additional data obtained are consistent with the existence of a sub-population of mitochondria that readily or constitutively exhibit the open conformation of the mPT pore *in vivo*. These data implicate further a systemic role for mutant huntingtin, and provide further evidence for a mitochondrial defect as a consequence of the gene mutation.

Activity of the tricarboxylic acid cycle component *α -ketoglutarate dehydrogenase complex (KGDHC)* is notably decreased in Alzheimer's and in several other neurodegenerative conditions. If this change is causally-linked to disease processes, then it is reasonable to expect that this linkage would be mediated by effects on mitochondrial physiology. In isolated rat forebrain non-synaptosomal mitochondria, inhibition of KGDHC exerts coincident effects on $\Delta\Psi$, Ca^{2+} transport, and Ca^{2+} retention as well as ruthenium red insensitive, Ca^{2+} -mediated loss of mitochondrial membrane potential. The latter phenomenon is conceptually similar to the changes associated with mPT. In isolated liver mitochondria, a system more amenable to mechanistic evaluation, inhibition of KGDHC facilitated mPT induction. This facilitation was independent of $\Delta\Psi$ during state 4 respiration, Ca^{2+} transport, and overall oxygen consumption. In contrast, progressive inhibition of respiration mediated by other substrates minimally affected or delayed mPT induction. These data suggest the potential for direct linkages between impaired KGDHC activity and neurodegenerative, in addition to cognitive, changes.



Interventions

While this evidence is consistent with induction of an mPT during neurodegeneration and neurological changes, it remains unclear whether mPT is on the causative pathway of cell death, or whether it is simply a downstream effect related to overall cellular collapse, which includes, for example, oxidative damage to components of the oxidative phosphorylation system. Although neuroprotection mediated by CsA was initially cited as evidence for causal involvement of mPT in ischemic injury, this is now appreciated to be problematic as CsA also affects calcineurin, the blockade of which itself has been shown to be neuroprotective. Similar "lack of specificity" arguments hold for other compounds, such as minocycline.

Minocycline is a second generation tetracycline antibiotic known to be protective in models of stroke, spinal cord injury, and neonatal hypoxia-reperfusion injury. While our recent work links minocycline to prevention of mPT-mediated release of mitochondrially-sequestered protein factors that facilitate both caspase-dependent and independent cell death pathways, other actions of minocycline have been identified, and the use of minocycline to build a case for mPT involvement awaits a more mechanistic study of the actions of minocycline. In addition, while minocycline appears to prevent mPT-mediated release of cytochrome c, protection is highly atypical and displays unexpected properties, including an associated loss of mitochondrial membrane potential, an apparently stoichiometric response, and a sharp, biphasic dose response.

Arguably, the best direct test of the hypothesis that mPT lies on the causative pathway of clinically-relevant cell death, at least in stroke, comes from the studies using N-Met-Val-CysA – a non-immunosuppressive analog of CsA reputed not to interact with calcineurin. This compound reduces infarct size in a rat model of transient focal ischemia (Friberg and Wieloch, 2002). The universal acceptance of mPT involvement in stroke remains limited however, at least in part, because of the reliance on data from a single drug, and the limited availability and characterization of its analog. Furthermore, even in stroke it appears that availability of CsA is limited by the blood brain barrier. Thus, there is a need to show that other characterized agents can modulate mPT induction and protect against cerebral infarction, both to answer this central mechanistic question in the pathogenesis of stroke-related neuropathology and to help reduce its clinical effects.

Over the past two years, we were one of thirty projects re-examining FDA-approved drugs. The purpose of these screens was to identify previously unknown activities of FDA-approved compounds so that these drugs might be moved rapidly into clinical trials to treat previously unexpected conditions. Our assay examined the ability of such compounds to inhibit the mPT. Screening identified a subset of neuroactive medicines, including tricyclics and phenothiazines, as being protective against mPT induction. Indeed, some of these medications have been in clinical use since the 1950's, are known to cross the blood brain barrier, and have been well-tolerated for long term use, despite their side effects. Initial screens have identified drugs that appear protective at doses approaching those in clinical use. Literature searches reveal data that some of these compounds (e.g., desipramine, trifluoperazine) exert cytoprotective effects *in vitro* and protect against ischemia-reperfusion in some animal models, supporting the potential for these drugs to be protective against excitotoxic injury. Because the side effects of different tricyclics vary, yet the protection appears mediated via similar mechanisms, it may be possible to use combinations of multiple tricyclics to reduce side effects while strengthening protection.

In summary, the mPT remains a plausible candidate for therapeutic intervention in stroke and other problems of both acute neurotoxicity and chronic neurologic neurodegeneration. Disease specific conditions that facilitate mPT induction may exist.



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Effect of Over-expression of Protective Genes on Mitochondrial Function of Stressed

Astrocytes

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Mitochondria are central to both normal cell function and the regulation of cell death. Within the brain astrocytes are crucial for neuronal metabolic, antioxidant, and trophic support, as well as normal synaptic function. In the setting of stress, such as during cerebral ischemia, astrocyte dysfunction may compromise the ability of neurons to survive. Despite their central importance, the response of astrocyte mitochondria to stress has not been extensively studied. Limited data already suggest clear differences in the response of neuronal and astrocytic mitochondria to oxygen-glucose deprivation. Measurement of mitochondrial enzymatic activity by Almeida et al. (Almeida et al., 2002) demonstrated oxygen glucose deprivation (OGD) induced mitochondrial dysfunction in neurons at duration that left astrocytes activity intact. Loss of astrocyte mitochondrial membrane potential with OGD was not associated with irreversible injury, but required about 1 h to recover once oxygen and glucose were restored (Reichert et al., 2001).

Prominent mitochondrial alterations during stress that can contribute to cell death include reduced ATP production, increased production of reactive oxygen species (ROS), and release of death regulatory and signaling molecules from the intermembrane space. In response to stress mitochondrial respiratory function and membrane potential can also change, and these changes depend in part on cell type. Bcl-2 family proteins are the best-studied regulators of cell death, and mitochondria are a major site of action for these proteins (Gross et al., 1999). The Bcl-2 family is divided into anti-apoptotic members including Bcl-2 and Bcl-x_L and pro-apoptotic members such as BAX and BID (Adams and Cory, 1998; Tsujimoto, 1998; Tsujimoto and Shimizu, 2000a; Tsujimoto and Shimizu, 2000b). Although much data supports the role of Bcl-2 family proteins in the regulation of some of these mitochondrial alterations, this remains an area of active investigation.

To better understand the ability of Bcl-x_L to protect astrocytes we examined mitochondrial function early after the imposition of oxidative stress. We previously reported that over-expression of Bcl-x_L increases astrocyte survival after both hydrogen peroxide exposure and glucose deprivation (GD) injury (Xu et al., 1999a). Here we describe our recent findings on the effects of Bcl-x_L on accumulation of reactive oxygen species (ROS), cell respiration and mitochondrial membrane potential in astrocytes exposed to hydrogen peroxide or GD. Overexpression of Bcl-x_L or a control gene was achieved in primary astrocyte cultures from mouse cortex using retroviral vectors (Xu et al., 1999a). This resulted in cultures in which essentially all the cells express the gene of interest.

Tetramethylrhodamine ethylester (TMRE), a potentiometric fluorescent dye that incorporates into mitochondria was used at 100 nM to determine the time course of changes in mitochondrial membrane potential (Ouyang et al., 2002). Distinct changes in astrocyte mitochondrial membrane potential were observed in response to H₂O₂ exposure as compared to GD. H₂O₂ induced a decrease in mitochondrial membrane potential (Ouyang et al., 2002), while GD caused an initial increase (at about 3 h, Table 1, Fig. 1) followed by a decrease (between 4 and 5 h) (Ouyang et al., 2002). Although both injuries involve oxidative stress, peroxide exposure may more rapidly damage oxidation sensitive mitochondrial proteins. Both the transient increase in the mitochondrial membrane potential after 2-3 h of glucose deprivation, and subsequent decrease in mitochondrial membrane potential were prevented by Bcl-x_L over-expression (Fig. 1 and (Ouyang et al., 2002).



Table 1. Effect of Bcl-x_L over-expression on astrocyte mitochondrial membrane potential^{with} glucose deprivation

	Control, -G	Lac-Z, -G	Bcl-x _L , -G	Control, +G
0 h GD	1.00±0.02	1.00±0.03	1.00±0.02	1.00±0.01
3 h GD	1.25±0.03*	1.26±0.04*	0.98±0.03 [#]	1.00±0.01 [#]
5 h GD	0.84±0.01*	0.80±0.02*	0.96±0.02 [#]	0.41±0.01 ^a

TMRE mitochondrial fluorescence with glucose deprivation (-G) in uninfected, Lac-Z, and Bcl-x_L over-expressing astrocytes was normalized to the basal fluorescence for each cell at the start of the experiment. Values are means ± SD of at least 50 astrocytes per condition. [#] indicates P<0.05 compared with the Lac-Z control at the same time, * indicates P<0.05 compared with 0 h same condition by ANOVA followed by Scheffe's test. ^a CCCP (5 μM) was used to induce a collapse of mitochondrial membrane potential in control cultures not deprived of glucose (+G).

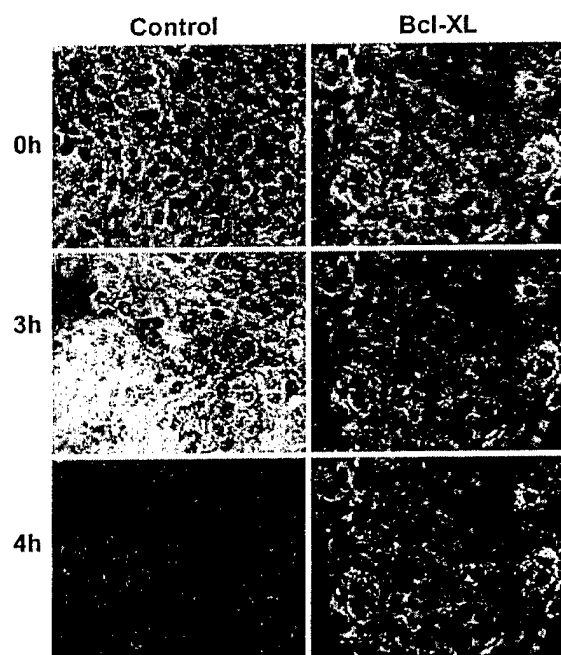


Figure 1.
TMRE was used to image mitochondrial membrane potential in astrocytes subjected to GD. Pseudocolor images at 0 h (upper panel), 3 h (middle panel) and 5 h (lower panel) of control uninfected (left column) and Bcl-x_L over-expressing (right column) astrocytes are shown. Hyperpolarized mitochondria are yellow to red, normal potential yellow to green, depolarized green to blue.

Oxygen consumption was measured in cortical astrocytes after increasing durations of GD (Ouyang and Giffard, 2003) using a modification of the method of Fiskum and colleagues (Fiskum et al., 2000; Moreadith and Fiskum, 1984). Astrocytes were permeabilized by adding 0.01% digitonin and state III respiration was initiated by adding 0.4 mM ADP. Oligomycin (2 μg/ml) was used to initiate state IV respiration and the uncoupled rate was determined by addition of carbonylcyanide *m*-chlorophenylhydrazine (CCCP, 0.1 μM). State III respiration decreased significantly as early as 3 h after removal of glucose (Table 2). At this time point state IV respiration and uncoupled respiration did not change. After 5 h of GD state IV respiration increased significantly and state III respiration declined further. In contrast, uncoupled respiration did not change much compared with 0 h GD. Although over-expression of Bcl-x_L did not



change basal respiratory rates (Ouyang and Giffard, 2003), when astrocytes were stressed, Bcl-x_L overexpression prevented the decrease in state III respiration and moderated the increase in state IV respiration (Table 2).

Table 2. Mitochondrial respiration of astrocytes during glucose deprivation

<i>Lac-Z</i>	<i>State III</i>	<i>State IV</i>	<i>Uncoupled</i>
0 h GD	25.2±3.1	5.1±0.4	35.9±3.6
3 h GD	18.6±2.3*	4.9±0.3	34.8±4.2
5 h GD	16.4±1.2*	8.1±0.5*	31.1±3.3
<i>Bcl-x_L</i>			
0 h GD	27.8±2.5	5.5±0.3	38.9±2.5
3 h GD	29.9±1.6 [#]	5.7±0.3 [#]	40.4±2.6
5 h GD	28.3±2.3 [#]	6.9±0.4 ^{##}	35.7±2.1

Values are means ± SD nmol O₂/min/mg proteins. # indicates P<0.05 compared with the Lac-Z control at the same time, * indicates P<0.05 compared with 0 h same condition by ANOVA followed by Scheffe's test.

Using the ROS-sensitive fluorescent dye hydroethidine, we recently demonstrated that with GD or H₂O₂, cultured astrocytes showed immediate and rapid increases in ROS accumulation which were markedly reduced by over-expressing Bcl-x_L (Ouyang et al., 2002). We previously found that peroxide exposure was associated with an increase in intracellular calcium as measured with Fura-2 (Ouyang et al., 2002). However, the improved survival with Bcl-x_L overexpression at 400 μM peroxide did not correlate with a reduction in the increase in intracellular calcium observed (Ouyang et al., 2002).

Fluorescence immunocytochemistry was performed to detect cytochrome c release (Ouyang and Giffard, 2003). At 3 h GD cytochrome c is localized in mitochondria while at 5 h about 1/4 of control astrocytes showed an evenly distributed immunostaining pattern demonstrating release of cytochrome c from the mitochondria to the cytosol. Mitochondrial morphology changed from elongated to punctuate in those cells. We observed that Bcl-x_L overexpression prevented loss of cytochrome c from mitochondria and the change in mitochondrial morphology (Ouyang and Giffard, 2003). We assessed cell death at these times by staining with Hoechst dye 33258 and propidium iodide. While essentially no cells stained with propidium iodide at the beginning of the experiment, at 3 h of GD 4.2±0.1% and after 5 h GD 15.4±0.4% of the control cells stained with propidium iodide. This suggests that the control-injured cells releasing cytochrome c are dead or dying. GD induced astrocyte death is more rapid in cells being imaged than in sister cultures maintained in the dark.

We conclude that in addition to the well-established ability of the anti-apoptotic Bcl-2 family members to block release of apoptotic factors from mitochondria, Bcl-x_L also improves mitochondrial respiratory function, normalizes membrane potential, and reduces production of free radicals by astrocytes subjected to oxidative stress. These improvements in astrocyte mitochondrial function may in part explain the ability of Bcl-x_L overexpressing astrocytes to protect wild type neurons co-cultured with them against GD and OGD (Xu et al., 1999b).



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Astrocyte mitochondria in *in vitro* models of ischemia

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Abstract

There is growing evidence that preservation of mitochondrial respiratory function during cerebral ischemia-reperfusion predicts the ultimate extent of tissue injury. Because neurons are selectively vulnerable to ischemic injury, many studies have focused on neuronal mitochondrial dysfunction in ischemia. However, positron emission tomography (PET) studies in animals and humans suggest that non-neuronal cells such as astrocytes may also experience mitochondrial metabolic compromise that contributes to ischemic necrosis. Astrocytes carry out a number of functions that are critical to normal nervous system function, including uptake of neurotransmitters, regulation of pH and ion concentrations, and metabolic support of neurons. Mitochondria are important for many of these actions. We have used a cell culture model of stroke - oxygen-glucose deprivation (OGD) - to study the response of astrocyte mitochondria to ischemia, and to evaluate how changes in astrocyte mitochondrial function might impact on neuronal survival and recovery after ischemia.

Introduction

Over the past decade, the central role that mitochondria plays in diseases of the nervous system has become increasingly clear (3), (5), (6). Mitochondrial functional abnormalities have been linked to genetic neurological disorders and neurodegenerative diseases (6). Mitochondrial dysfunction has also been implicated in ischemic injury, in which inadequate delivery of oxygen and glucose limits mitochondria respiration. Recent studies suggest that, in fact, the degree of mitochondrial dysfunction in cerebral ischemia may be a critical determinant of the final extent of tissue injury.

Studies on mitochondria isolated from ischemic brain suggest that ischemia-reperfusion can cause short-term and long-term alterations in mitochondrial function (7), (8), (9), (10). After brief focal ischemia, isolated mitochondria demonstrate defects in State 3 (ADP-dependent), and State 4 (ADP-independent) respiration. These deficits resolve after a brief period of reperfusion. Longer periods of ischemia lead to prolonged impairment in State 3 respiration (10). In addition, although mitochondrial metabolism normalizes soon after ischemia-reperfusion, many studies have found a secondary decline minutes to hours later (11), (12), (13). The cause of this delayed decline is proposed to be oxidative damage to mitochondria during reperfusion (12), (14), or the effect of fatty acids released during ischemia on mitochondrial function (15), (16). Decreased pyruvate dehydrogenase (PDH) activity is also impaired after ischemic-reperfusion (17), (18).

The selective vulnerability of neurons to ischemic injury has been taken as an indication that neurons experience greater metabolic deterioration than astrocytes, which are relatively resistant to ischemia injury. Astrocytes also contain glycogen stores, which are presumed to allow them to maintain ATP production through glycolysis and mitochondrial membrane potential by reversal of the F_0F_1 -ATPase. Although loss of mitochondrial membrane potential has been documented in neurons exposed to ischemic conditions, as will be discussed, astrocytes also exhibit early mitochondrial depolarization when exposed to oxygen-glucose deprivation (OGD).

ASTROCYTE MITOCHONDRIAL DEPOLARIZATION DURING OGD

The *in vitro* oxygen-glucose deprivation model used for our studies has previously been described in detail (19), (20). In this model, mixed cortical astrocyte-neuronal co-cultures are exposed to oxygen-glucose deprivation (OGD) for 30-60 minutes. Fifty percent of neurons are irreversibly injured after 45 minutes of OGD, with 100% neuronal death produced by 60-70 minutes of OGD. In contrast, astrocytes in the same cultures are resistant to OGD-induced death, requiring



4 h for irreversible injury. Previous work with this model has found that, even after 60 minutes of OGD, ATP levels are still 70% of control levels. In addition, benzodiazepines and barbiturates worsen neuronal cell death through effects on mitochondrial function, reproducing findings in stroke patients receiving these two classes of agents, who have a worse prognosis, and suggesting that this *in vitro* model mimics many of the metabolic aspects of cerebral ischemia.

Using this system, we found that exposure to 45-50 minutes of OGD produced a 70% decrease in mitochondrial membrane potential (ψ_m) in astrocytes, determined by confocal imaging of tetramethylrhodamine ethyl ester fluorescence (2). This time point correlates with the previously-reported rise in extracellular glutamate and with the onset of irreversible injury to neurons observed in this model (19). In our experiments, treatment with a nitric oxide synthase inhibitor (G N-Arg) partially blocked the decline in mitochondrial membrane potential (ψ_m), suggesting that nitric oxide (NO) or peroxynitrite were involved in loss of ψ_m . Brown and Borutaite (1999) have previously found that both NO and peroxynitrite can inhibit mitochondrial electron transport chain activity at multiple sites. We were unable to differentiate between a role for NO vs. peroxynitrite in our model, but given the low O_2 (0.2 %) in the anaerobic chamber used for these experiments, it is unlikely that an extensive amount of peroxynitrite would be present.

Astrocyte ψ_m was also preserved during OGD by treatment with cyclosporine A, indicating that opening of the mitochondrial permeability transition pore (mtPTP) is involved in loss of astrocyte ψ_m . Assembly of the mtPTP is triggered by several stimuli, including fatty acids, accumulation of mitochondrial calcium, and oxidative stress, events that are reported to occur during ischemia-reperfusion injury.

In the absence of OGD, astrocytes are clearly capable of using ATP to maintain ψ_m , by reversal of the F_0F_1 -ATPase to support ψ_m but why astrocytes exposed to OGD fail to support ψ_m through this mechanism is not clear. Involvement of glutamate receptor-mediated calcium entry and direct uncoupling by Ca^{2+} are unlikely because blocking AMPA/kainate receptors failed to protect astrocyte ψ_m (2). Co-incubation with 1% bovine serum albumin (BSA, fatty-acid free) also failed to modify ψ_m loss (Reichert and Dugan, unpublished), suggesting that fatty acid release from neurons is also not likely to be involved. Loss of ψ_m may involve glutamate, acting through the transporter, or adenine nucleotides released by neurons (Fig. 2). This may add to the growing body of literature describing extensive communication between neurons and astrocytes (21), (22), (23).

Recovery of astrocyte ψ_m after reintroduction of O_2 and glucose was a gradual process, requiring > 1 h. There were also ultrastructural changes in mitochondria exposed to OGD that persisted for a relatively prolonged period of time suggesting that OGD causes specific but reversible changes in astrocyte mitochondrial physiology beyond lack of O_2 and glucose (Fig. 1). CsA decreased the extent of morphological changes. It is possible that these changes may correspond to the mitochondrial swelling and matrix alterations reported in early post ischemic brain using electron microscopy (24).

IMPLICATIONS OF ASTROCYTE MITOCHONDRIAL DYSFUNCTION

There are a number of potential downstream effects of mitochondrial depolarization. In most cell types, such prolonged loss of ψ_m would activate apoptotic pathways and result in cell death. Depolarization might also lead to loss of intramitochondrial contents, such as ADP, leading to prolonged impairment in mitochondrial respiration and ATP production. If depolarization altered the production of reactive oxygen species, such as H_2O_2 , this might result in detrimental changes in intracellular and intercellular signaling through redox-sensitive pathways, such as Ras, Erk1/2, and NF κ B. We have been exploring each of these potential outcomes in turn (Fig. 2).

Initiation of the Apoptotic Cascade

Activation of the mtPTP can lead to mitochondrial depolarization and is associated in many cell types with release of cytochrome c from the inner mitochondrial membrane (25). The free



cytochrome c may then activate caspase 9 through binding to apaf-1. Caspase 9, which resides in the mitochondrial matrix (as a pro-caspase), may be released when the barrier function of the inner membrane is lost, and can activate and work synergistically with caspase 3 to trigger downstream effectors of apoptosis (26).

We observed a 20% loss of cytochrome c from mitochondria at the end of OGD that was partly blocked by CsA. However, release of cytochrome c was not accompanied by activation of either caspase-9 or caspase-3, suggesting that cytochrome c was blocked from activating the mitochondrial caspase cascade.

Decreased energy production and altered ion homeostasis

In addition to its role in apoptosis, release of cytochrome c from mitochondria could alter mitochondrial function by hindering the efficient transfer of electrons through cytochrome aa₃ in cytochrome oxidase, enhancing upstream superoxide radical production from ubiquinone redox cycling (6). Elimination of ψ_m abolishes mitochondrial Ca²⁺ uptake, and may impair many other aspects of mitochondrial metabolism, in addition to the most well-known - impairment of ATP production. Extensive data indicate that astrocytes are involved in a number of processes that affect neuronal survival, such as glutamate uptake, maintenance of extracellular pH and potassium, Ca²⁺ buffering, and transfer of lactate and/or pyruvate to neurons as energy substrates (22), (27), (28), (29), (30). A number of these functions are dependent on mitochondrial membrane potential, and have been reported to be impaired early in ischemia (31), (32), (33).

Altered ROS production and effects on signaling

One additional effect that mitochondrial depolarization be altered mitochondrial ROS generation. Whether ROS production would be enhanced or decreased by OGD-reperfusion is not entirely clear, and might vary with the duration of OGD, and the time after reperfusion. Mitochondria might be one source, but NADPH-depleted NOS might also contribute. Further work on the source(s) and timing of astrocyte ROS production during OGD is ongoing.

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radical (34), (35), nitric oxide, and peroxynitrite (36), act as signaling molecules to regulate kinase cascades, transporters, ion channels, and transcription factors. Our recent studies indicate that OGD alters activity of several signaling pathways, including MAPK (Fig. 2). To what extent changes in MAPK activity alter astrocyte and neuronal gene expression and function is an area of continuing investigation.

SUMMARY

Depolarization of astrocyte mitochondria during OGD might have both beneficial and harmful effects on neuronal survival. Short-term loss of ψ_m would allow astrocytes to shift use of glycogen and glucose temporarily away from aerobic metabolism to glycolysis, increasing the amount of lactate available for delivery to metabolically impaired neurons (Fig. 2). This might be tolerated for as long as astrocyte glycogen stores were available. However, prolonged loss of ψ_m in astrocytes might be expected to have injury-promoting effects during CNS ischemia. Astrocytes are involved in the normal maintenance of brain homeostasis, including several energy-dependent functions necessary for normal neuronal activity, e.g., regulation of extracellular K⁺, pH, and osmolality, export of metabolic intermediates, and rapid uptake of neurotransmitters (21), (22), (30). The ability of astrocytes to maintain these functions may, in fact, be a critical determinant of neuronal survival after ischemia (33), (37), (38), (39). Furthermore, metabolic imaging studies have suggested that mitochondrial function in post-ischemic brain may be impaired for hours-to-days after the ischemic insult (40), (41), (42), (43), (44). Loss of ψ_m and eventual energy failure in astrocytes might lead to an inability to provide these critical support functions during ischemia, thus



exacerbating ischemic injury to neurons. Loss of ψ_m and eventual energy failure in astrocytes might lead to an inability to provide these critical support functions during ischemia, thus exacerbating ischemic injury to neurons. Our data suggest that therapies targeted at astrocyte mitochondria might act synergistically with neuron-based strategies to provide protection to the ischemic brain.

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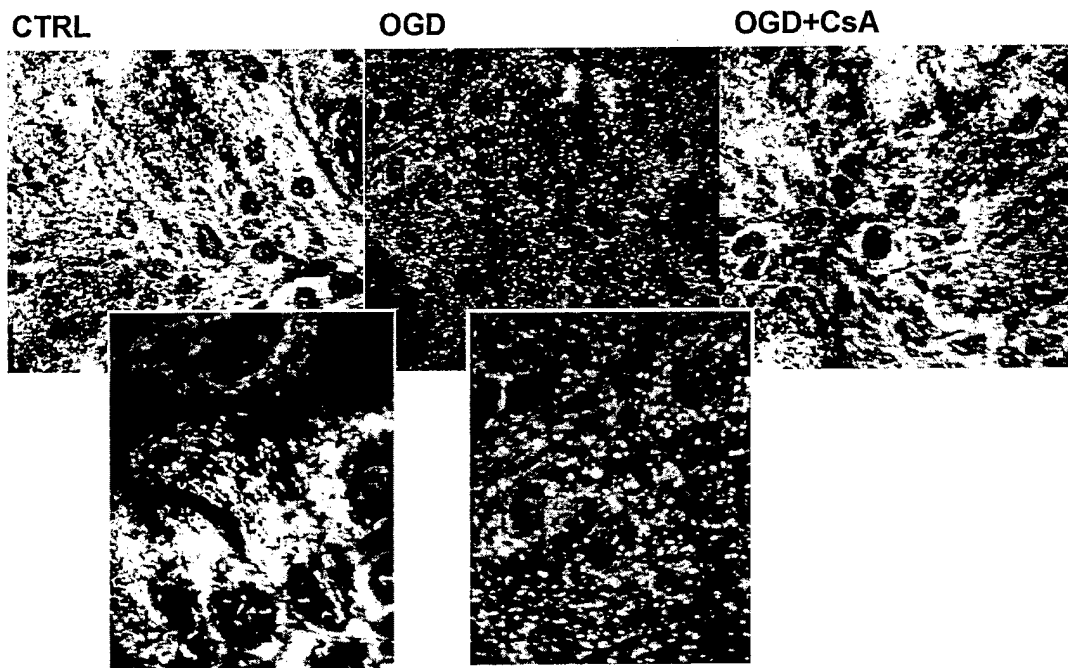


Figure 1 Fluorescence confocal images of astrocyte mitochondria after OGD. Mixed cortical cultures were loaded with TMRE (50 nM) and exposed to 50 minutes OGD. Panels show control, OGD, and OGD plus cyclosporine A (10 μ M) conditions. Insets are at 4X magnification.

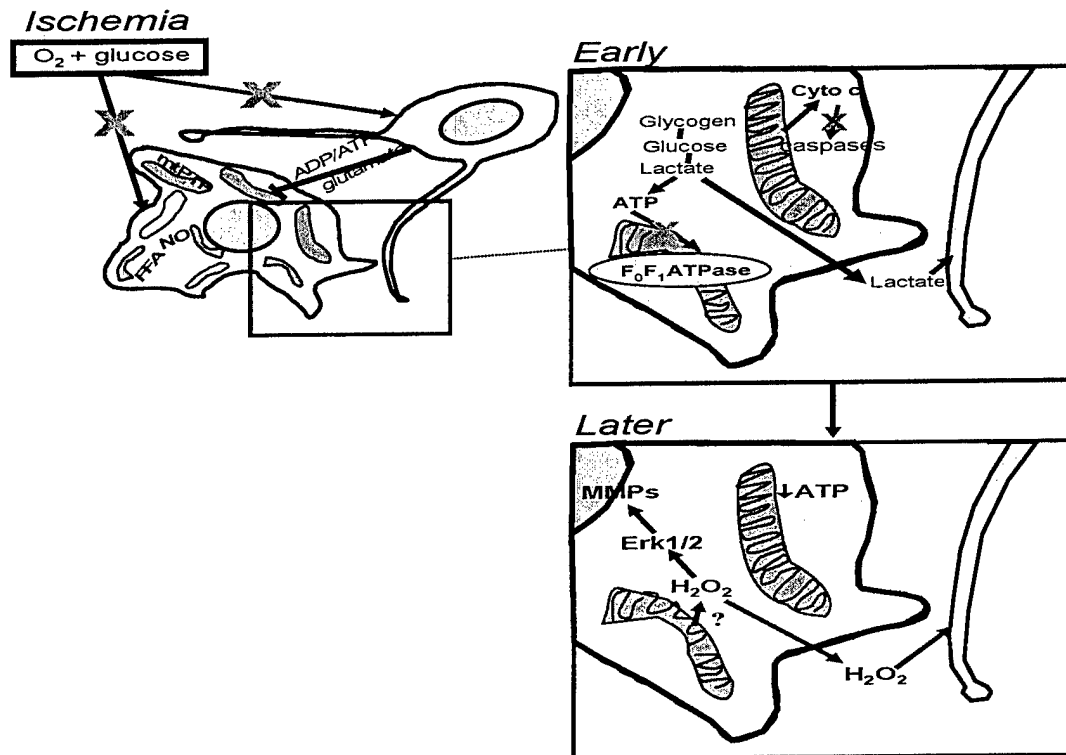


Figure 2 Proposed sequence of astrocyte mitochondrial changes and secondary effects after OGD. Abbrev: NO=nitric oxide, FFA=free fatty acid, mtPTP=mitochondrial permeability transition pore, MMP=metalloprotease. Decreased availability of O_2 and glucose is sensed by neurons and astrocytes. Astrocyte mitochondria depolarize, a process accelerated by a soluble factor from neurons (Kim-Han, unpublished), which might be glutamate through the transporter (1), or adenine nucleotides. Loss of ψ_m involves NO, the mtPTP (2), and possibly fatty acids (4). ATP levels are preserved, but ATP is not used to support ψ_m , presumably due to block at the F_0F_1 ATPase. This could allow shunting of lactate to metabolically-compromised neurons. Cytochrome c is released but fails to activate caspases. The ratio of cytochrome c to Inhibitor of Apoptosis Proteins (IAP), which appears to be lower in astrocytes, might be partially responsible (Dugan and Kim-Han, unpublished). At later time points, levels of H_2O_2 in astrocytes increase, modifying redox-dependent signaling pathways, such as Erk1/2. Potential sources include the ETC or NADPH-depleted NOS. Low levels of H_2O_2 might also be released from astrocytes to alter redox-sensitive systems in neurons.



Neuroprotective Effects Of Ischemic Preconditioning in Brain Mitochondria Following Cerebral Ischemia

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The mechanisms leading to neuronal cell death after cerebral ischemia are complex. A well established fact in this field is that cells continue to die over months after a stroke, a phenomena that has been termed delayed cell death. Although not clearly defined, neuronal cell death may result from either apoptosis, necrosis or a cell death mechanisms that is a mixture of these processes [1, 2].

Numerous studies support the hypothesis that reperfusion following cerebral ischemia contributes substantially to ischemic injury [3-6] and that mitochondrial dysfunction plays a central role [7-13].

Evidence of mitochondrial dysfunction following cerebral ischemia was described in previous studies, as a prominent change in redox activity of mitochondrial respiratory chain components in post-ischemic brain [14-19]. This hyperoxidation of electron carriers is indicative of either a response to decreased substrate availability [14] and/or a reaction of mitochondrial complexes to reactive oxygen species (ROS) [15]. Post-ischemic mitochondria may also be a major source of ROS, and free radical-mediated damage has been linked to reperfusion injury following brain ischemia [20-25]. However, recent findings suggest that this hyperoxidation may result from loss of electron carriers from mitochondria following cerebral ischemia, such as cytochrome c and NADH [26]. The loss of cytochrome c from mitochondria might affect respiratory chain activity and/or it may trigger the apoptotic cascade [27, 28]. This is suggested by findings that apoptosis (programmed cell death) may be linked to mitochondria and their release of cytochrome c [10, 11, 29, 30].

Additional evidence of mitochondrial dysfunction was described in studies from isolated brain mitochondria. Mitochondria isolated from ischemic brain exhibited decreases in state 3 respiratory rates of approximately 70% with NAD-linked respiratory substrates [31]. Cafe et al. [32] showed that non-synaptosomal mitochondria were insensitive to ischemia but that they became dysfunctional in the late reperfusion phase. Mitochondria from synaptic terminals were greatly affected by ischemia but partially recovered during reperfusion. Sims and Pulsinelli [33] also reported that in rat a model of forebrain transient ischemia the rate of oxygen consumption decreased in the CA1, CA3 and CA4 regions in the late reperfusion phase. This study was performed in homogenates from different brain sub-regions.

Ischemic preconditioning:

Ischemic preconditioning (IP)

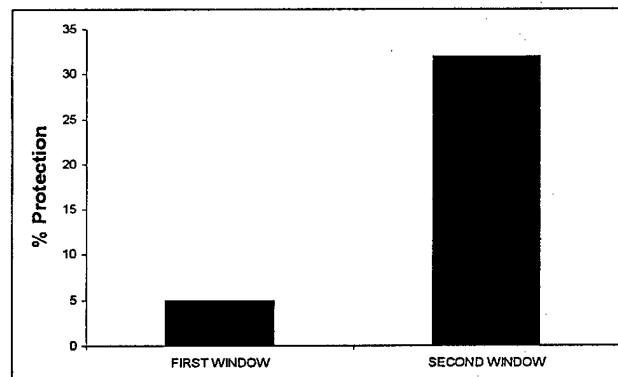


Figure 1: Comparison between the % protection in the two windows of ischemic preconditioning. In the first window, IPC preceded the test ischemic insult by 30 min. In this window, IPC protected against histopathology at 3 days, but not 7 days of reperfusion. In contrast, when IPC preceded the test ischemic insult by 48 h, there was approximately 33 % of normal neurons after 7 days of reperfusion.



refers to the ability of a brief ("sublethal") ischemic episode, followed by a period of reperfusion, to increase an organ's resistance to injury (ischemic tolerance) following a subsequent ischemic event. This induction of tolerance against ischemia resulting from sublethal ischemic or anoxic insults has gained attention as a robust neuroprotective mechanism against conditions of stress such as anoxia/ischemia in heart and brain [34-40]. There are different preconditioning paradigms both in heart and brain. Among variations in preconditioning paradigms include, the number of preconditioning insults, types of preconditioning insults, and time between preconditioning and the test insults.

In the past, most preconditioning studies in brain have suggested that several hours are required to develop the tolerant state. However, in recent studies we and others suggested that preconditioning with a rapid onset time course, similar to that in heart (within 1 h), can protect synaptic activity after anoxia in brain slices [41-46] and reduce histopathology after ischemia in intact brain [47-51].

Thus, two windows have been identified, one that occurs very rapidly (within 1 h) [47-49] and a second one that develops slowly (over days) [40]. A difference between these 2 windows is that neuroprotection in the first window is transient. We observed that significant neuroprotection against histopathology was evident after 3 d of reperfusion, but after 7 d, histopathology was similar between controls and preconditioned rats [47]. In contrast, when rats were preconditioned days prior to the 'test' ischemic insult, the neuroprotection was robust and long lasting [40, 52].
IPC and mitochondrial protection in the first window

Recent studies from this laboratory and others suggest that preconditioning from the first window can protect ischemic injury, as assessed by the release of lactic dehydrogenase (LDH) or by synaptic activity recovery after the test insults in cell cultures [53], brain slices [41-46] and reduce histopathology after ischemia in intact brain [47, 49-51, 54]. However, protection only ensued if reperfusion was allowed to occur for 3 d but not beyond [47]. Thus, it was important to ascertain whether this lack of chronic protection was due to less protection against mitochondrial dysfunction by IPC.

Indeed, we found that the first window of IPC could not protect mitochondria against the deficits in respiration through complexes I – IV [55]. Rates of respiration in presence of pyruvate + malate and succinate + glycerol – 3 – phosphate decreased in both the ischemia and in IPC groups marginally. However, significant decreases in the rate of respiration for complex IV were observed in both the ischemia and IPC groups. This decrease in the rate of respiration in the presence of complex IV substrates is suggestive of impairment in oxidative phosphorylation. This decrease in the rate of respiration at the level of complex IV was not accompanied by significant decreases in complex IV activity. A possible explanation for the decrease of respiration in complex IV and no change in complex IV activity in the ischemia group is that cytochrome c may be released from mitochondria following test ischemia. This contention is supported by previous studies. We found that the apparent mitochondrial hyperoxidation linked to brain dysfunction may be caused by disruption of the mitochondrial membrane and the concomitant loss of the mitochondrial electron carriers [26]. In that study, cytosolic cytochrome c was increased following global cerebral ischemia and 30 min of reperfusion (same model of cerebral ischemia as in the current study); and conversely, reducible cytochrome c (presumably the intramitochondrial fraction of this cytochrome) was decreased following anoxia in hippocampal slices. This latter finding was correlated with NADH hyperoxidation that occurs following anoxia in hippocampal slices and the first window of preconditioning was unable to protect against such hyperoxidation [44].
IPC and mitochondrial protection in the 2nd window

In contrast to the lack of mitochondrial protection by IPC in the first window, IPC in the second window significantly protected mitochondria against the deficits in respiration through complexes I – IV [56]. As described above, many studies have demonstrated that reactive oxygen species (ROS) and the resulting oxidative stress play a pivotal role in neuronal cell death [20-25].



There are two major regions in the electron transport chain where ROS are produced. One is complex I and the other complex III [57]. Since oxidative stress is implicated in the pathophysiology that ensues after cerebral and cardiac ischemia [58], one can surmise that a key mechanism by which IPC in the second window protects hippocampus against delayed neuronal cell death is by protecting mitochondrial oxidative phosphorylation.

However, the precise mechanism by which IPC affords protection to mitochondria remains undefined. A possible mechanism may involve up-regulation of neuroprotective genes. Cai and Storey [59] found that anoxia stress induces up-regulation of the genes for NADH-ubiquinone oxidoreductase subunit (encoded by mitochondrial gene) and cytochrome oxidase subunit 1 (encoded by mitochondrial gene) in the anoxic-resistant turtle heart [59]. We presume that IPC may up-regulate certain genes responsible for the activities of these complexes, which may render hippocampal mitochondria resistant to 'lethal' ischemia.

Another possible mechanism may involve better maintenance of ATP. There is considerable evidence for the role of ATP depletion, which follows hypoxic/ischemic insults, in the development of mitochondrial damage and the subsequent activation of downstream cell death pathways [60]. Yabe et al. [61] have shown that in preconditioned heart, the glycolytic ATP production increases. They have also demonstrated that ATP and creatine phosphate concentration remained higher in preconditioned group as compared to non-preconditioned group [60]. A higher level of ATP in preconditioned group may prevent cytochrome c release from the mitochondria, as was previously observed by Galeffi et al. [60]. Putative role of the signal transduction pathway on mitochondrial protection after cerebral ischemia: role of protein kinase C (PKC)

We recently demonstrated that one specific PKC isozyme, namely ϵ PKC, plays a pivotal role in the induction of tolerance after ischemic preconditioning [62-64]. Basu et al. [65] showed that cleavage of ϵ PKC by caspase-7 results in the activation of ϵ PKC, which was associated with its anti-apoptotic function. Also, formation of mitochondrial ϵ PKC-ERK $\frac{1}{2}$ modules was coupled to the inactivation of BAD, a pro-apoptotic molecule [66]. Since, ischemic preconditioning has been shown to preserve mitochondrial function [56, 67], we hypothesize that ϵ PKC promotes ischemic tolerance by protecting mitochondrial function during the reperfusion phase. However, the precise mechanism by which IPC via ϵ PKC protects mitochondria following cerebral ischemia remains undefined. Further studies from our laboratory are underway to define these mechanisms.

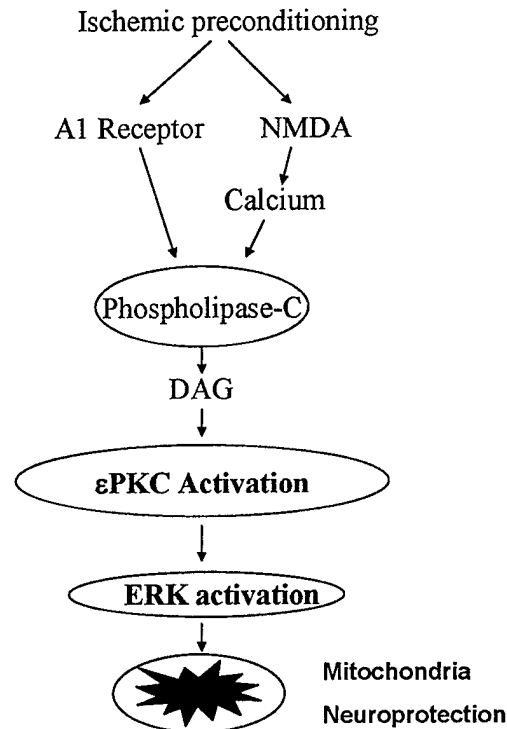


Figure 2: Flow chart defining the signal transduction pathways leading to ischemic tolerance. We and others have characterized that the adenosine A1 and the NMDA receptors are involved in the triggering phase of IPC. The ensuing pathways lead to ϵ PKC and ERK activation, which we propose may be protecting mitochondria.



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Mitochondrial glutathione: a modulator of brain cell death

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Introduction

Glutathione in cells is a major antioxidant acting both directly to remove reactive oxygen species and as a substrate for several peroxidases (Dringen, 2000). This tripeptide is also involved in other reactions including the conjugation of foreign molecules catalyzed by glutathione S-transferases. Cellular glutathione is found in two separate but interacting pools located in the cytoplasm and mitochondria. The cytoplasmic pool typically accounts for 85% or more of the total glutathione in cells (Griffith and Meister, 1985; Meister, 1995; Lash et al., 1998). Most, if not all, of the synthesis of glutathione occurs in the cytoplasm (Griffith and Meister, 1985; Martensson et al., 1990; Lash et al., 1998). Thus, the long-term maintenance of mitochondrial glutathione depends on transport from this site.

Mitochondrial glutathione and cell viability

In many cells, glutathione in the mitochondria is much more important than the larger cytoplasmic pool in maintaining cell viability and limiting damage to various potentially toxic treatments. This key contribution of mitochondrial glutathione in preserving cell viability was first proposed by Meredith and Reed (1982, 1983) based on the greatly increased death of hepatocytes when both glutathione pools were experimentally depleted compared to cells with losses of cytoplasmic glutathione alone. Subsequent studies identified similar responses in other types of cells and further showed that depletion of glutathione from the mitochondria was associated with much greater dysfunction and loss of viability in cells challenged with a range of oxidative stresses and other insults (Fernandez-Checa et al., 1991; Dhanboora and Babson, 1992; Shan et al., 1993; Colell et al., 1998, 2001; Wullner et al., 1999). In many of the relevant investigations, the deleterious response to mitochondrial glutathione depletion was demonstrated on a background of cytoplasmic glutathione loss making it difficult to unequivocally evaluate the contribution of the mitochondrial pool alone. Selective partial depletion of mitochondrial glutathione has been achieved without accompanying changes in the cytoplasmic pool in only a few studies. Such cells also exhibited substantially decreased viability in response to substances including t-butylhydroperoxide (Fernandez-Checa et al., 1991) and tumor necrosis factor (Colell et al., 1998, 2001) providing more direct evidence of the essential role of mitochondrial glutathione in preserving cell function.

These findings point to the potential for mitochondrial glutathione changes to contribute to tissue dysfunction and damage in disease states. Partial losses of mitochondrial glutathione develop *in vivo* under some conditions that include long-term ethanol feeding (Fernandez-Checa et al., 1991) and liver ischemia with reperfusion (Grattagliano et al., 1999). Glutathione loss induced in the liver by ethanol feeding is restricted to the mitochondrial pool. It results from a decreased glutathione uptake into mitochondria due to changes in the fluidity of the mitochondrial membranes (Fernandez-Checa et al., 1991; Colell et al., 1998, 2001; Coll et al., 2003). This partial loss of glutathione increases the susceptibility of the liver cells to several potentially toxic treatments (Fernandez-Checa et al., 1991; Colell et al., 1998, 2001; Zhao et al., 2002).



Interactions of mitochondrial and cytoplasmic glutathione

The mechanisms of glutathione uptake into mitochondria and the control of this process have mostly been investigated in organelles isolated from the liver and kidney. The relevance of the findings to other tissues including the brain remains to be established. Glutathione carries a net negative charge. Its transport into mitochondria involves movement against the charge gradient across the inner mitochondrial membrane and therefore requires either energy or exchange with another anion. In kidney mitochondria, the 2-oxoglutarate and dicarboxylate transporters appear to be major contributors to glutathione uptake (Chen and Lash, 1998; Lash et al., 2002). Transport into liver mitochondria shows some similarities but also significant differences in properties, (Martensson et al., 1990; Meister, 1995; Coll et al., 2003), suggesting tissue specific characteristics. The 2-oxoglutarate transporter again seems to be involved (Coll et al., 2003).

Although the mitochondria have an ongoing reliance on the cytoplasm for the supply of glutathione, these two pools can be separately modulated under some conditions. The glutathione synthesis inhibitor, buthionine sulfoximine, has been found in many different types of cells to substantially deplete cytoplasmic glutathione while having little initial impact on the mitochondrial pool (Martensson et al., 1989; Meister, 1995). Indeed, treatment with this compound in vivo typically causes major losses of the cytoplasmic pool within a few hours in a range of tissues but only produces slow depletion of mitochondrial glutathione over many days or weeks. Partial selective losses of the mitochondrial glutathione pool also develop in response to some treatments of cells in vitro (Shan et al., 1993; Collet et al., 1998; 2002) and in disease models in vivo (Fernandez-Checa et al., 1991; Wallin et al., 2000; Anderson and Sims, 2002). Thus, under the conditions examined in these studies, the transport processes do not replenish glutathione content in the mitochondria even though cytoplasmic glutathione is preserved.

Mitochondrial glutathione in cells from brain

Despite the evidence for the important roles of mitochondrial glutathione in non-neural cells, surprisingly little attention has been given to the properties and functions of this antioxidant pool in cells from the central nervous system. Cerebellar granule neurons in culture exhibit marked functional deterioration and die in response to complete loss of both the mitochondrial and cytoplasmic glutathione but not with cytoplasmic glutathione loss alone (Wullner et al., 1999). Thus, these neurons exhibit a similar dependence on mitochondrial glutathione to other cell types. Complete depletion of glutathione in motor neurons (Rizzardini et al., 2003) and in astrocytes in culture (Huang and Philbert, 1996) also promotes cell dysfunction and death, although these studies did not relate changes in cell vulnerability to the responses of the individual glutathione pools.

We have recently established conditions based on ethacrynic acid treatment of cortical astrocytes in culture that result in complete loss of mitochondrial glutathione while leaving the cytoplasmic pool essentially unchanged. Ethacrynic acid has been widely used to produce total glutathione depletion in various cells, including neurons and astrocytes in culture. This compound is conjugated with glutathione in both the cytoplasm and mitochondria in reactions catalysed by some glutathione S-transferases. In neurons and astrocytes (Huang and Philbert, 1996; Wullner et al., 1999), but not in some non-neural cells (Meredith and Reed, 1982), ethacrynic acid produces more rapid depletion of the glutathione in the mitochondria than in the cytoplasm. This differential selectivity has not previously been exploited experimentally. Indeed, as far as we are aware, there are no previous reports for any cell type in which comparable selectivity of mitochondrial glutathione depletion has been achieved. Thus, this preparation provides a valuable tool for assessing the function of mitochondrial glutathione in a major population of cells derived from the brain.

Astrocytes with depleted mitochondrial glutathione showed no change in viability compared with non-depleted cells when incubated under normal conditions for 24 h. However, the glutathione-depleted astrocytes were much more susceptible to exposure to the peroxynitrite donor,



3-morpholininosydnonimine (Sin-1) and to nitric oxide (Muyderman et al., 2004; Muyderman, Nilsson and Sims, submitted). The cells treated with Sin-1 exhibited earlier and larger changes in cell function compared with astrocytes with preserved mitochondrial glutathione and also showed a substantial increase in cell death based on lactate dehydrogenase release and propidium iodide staining at both 3 h and 24 h after treatment.

Interestingly, the glutathione content of the mitochondria recovered only partially over several hours following ethacrynic acid treatment despite the ongoing availability of glutathione in the cytoplasm. Thus, these cells showed much less rapid restoration of glutathione content than expected based on studies of isolated mitochondria from other tissues. This finding suggests either that the glutathione transport exhibits different properties in these cells or it has been modified as a result of the ethacrynic acid treatment. A slow recovery of partially depleted mitochondrial glutathione despite a preserved cytoplasmic pool has also been seen following treatment of COS cells with 4-hydroxynonenal (Raza et al., 2003). This compound is also conjugated in a reaction catalyzed by glutathione S-transferase raising the possibility that the products of such reactions might interfere with glutathione uptake into the mitochondria.

Mitochondrial glutathione in the astrocytes treated with ethacrynic acid could be fully replenished using glutathione monoethylester as a precursor. Importantly, such restoration of the mitochondrial glutathione blocked the increased cell death resulting from exposure to Sin-1, clearly implicating the glutathione loss (and not some other consequence of ethacrynic acid treatment) as the basis for the greater vulnerability of these cells. The cell loss was also blocked by incubations with cyclosporin A, suggesting a role for induction of the mitochondrial permeability transition in the increased susceptibility of glutathione-depleted astrocytes. The oxidation of specific protein sulfhydryls promotes induction of the permeability transition (Kowaltowski et al., 2001), providing a likely link between glutathione depletion and this deleterious mitochondrial change.

Tissue damage in stroke: a role for mitochondrial glutathione depletion?

In keeping with the relatively limited investigations of the role of mitochondrial glutathione in the central nervous system, there have been very few attempts to address whether mitochondrial glutathione is altered in neurological disorders. Transient selective depletion of glutathione in the mitochondria develops as an early change in a model of neonatal hypoxia-ischemia (Wallin et al., 2000). We have also identified decreases in this antioxidant pool in a model of stroke in adult rats and provided evidence suggesting that this change could contribute to the tissue damage that develops in this major neurological disease. These changes and their possible implications for the pathology associated with stroke are briefly reviewed below.

Stroke in humans most commonly arises from blockage of an intracerebral artery. Although this occlusion is usually long-lasting or permanent, temporary occlusion is also seen in a subgroup of patients, particularly with recent increased use of thrombolytic agents to reverse the arterial blockage. The focal cerebral ischemia resulting from arterial occlusion typically produces immediate functional impairment. Unless the occlusion is reversed within the first hour or so, changes are initiated that lead over many hours to tissue infarction due to the death of all cell types within parts of the perfusion territory of the affected vessel. The location and volume of infarcted tissue are important determinants of the long-term symptoms of stroke. Thus, there has been considerable effort in recent years to elucidate the molecular events contributing to the ischemia-induced cell loss with a view to identifying therapeutic targets to limit tissue damage and improve outcome (Lipton, 1999; Sims and Anderson, 2002; Zheng et al., 2003). These studies have provided evidence that interactions of multiple deleterious changes are required for cell death, with the exact mechanism being greatly influenced by factors including the severity of the ischemia and whether arterial occlusion is permanent or temporary.

Changes in mitochondrial function are one of the factors likely to contribute to ischemic cell death under at least some conditions (Sims and Anderson, 2002). The most direct mitochondrial



effects arise from the reduced delivery of oxygen to the tissue. The resultant impairment of the electron transport chain contributes to marked disruption of ATP and related metabolites in the most severely ischemic "core" or "focal" tissue within the perfusion territory of the affected vessel and also to lesser changes in surrounding perifocal tissue that is subjected to more moderate reductions in blood flow (Folbergrova et al., 1992, 1995). Many of these metabolite changes substantially recover on reperfusion even in tissue destined to become infarcted but lactate often remains elevated, suggesting ongoing mitochondrial impairment. Cell susceptibility may be further compromised by direct decreases in mitochondrial capacity for respiratory function which develop during ischemia and reperfusion (Kuroda et al., 1996; Nakai et al., 1997; Anderson and Sims, 1999). Increased production of nitric oxide and its derivatives has been strongly implicated as a contributor to tissue damage in stroke (Eliasson et al., 1999). One deleterious effect of these substances could result from their ability to inhibit the electron transport chain (Radi et al., 2002). More direct support for a mitochondrial contribution to cell death is provided by the substantial protection achieved using a mitochondrial potassium channel opener in both permanent and temporary ischemia (Liu et al., 2002; Shimizu et al., 2002) and also by inhibitors of the mitochondrial permeability transition administered near the time of reperfusion in temporary ischemia (Yoshimoto and Siesjo, 1999; Matsumoto et al., 1999).

We have identified a partial loss of glutathione in mitochondria isolated from ischemic brain regions in a rat model of stroke (Anderson and Sims, 2002). This change persisted for at least several hours of reperfusion. The glutathione losses during ischemia were not accompanied by changes in total tissue glutathione and were only seen with ischemic periods sufficient to induce subsequent infarction. Indeed, the time at which mitochondrial glutathione loss was first detected during ischemia in focal tissue from the striatum and cortex and in cortical perifocal tissue corresponded with the ischemic times typically required to initiate infarct formation in these same regions. This association is consistent with the mitochondrial glutathione loss being one factor contributing to the tissue damage, perhaps by providing conditions promoting induction of the permeability transition or other deleterious mitochondrial changes.

The glutathione depletion in ischemia was temporarily blocked or reversed by a single intracerebral injection of glutathione monoethylester but this treatment did not modify infarct volume (Anderson et al., 2004a). However, more prolonged treatment with this glutathione ester via intracerebroventricular infusion initiated at the time of temporary arterial occlusion reduced infarct volume by more than 60% (Anderson et al., 2004b). Further studies are needed to demonstrate that mitochondrial glutathione is indeed contributing to this protective response and to identify the critical period(s) during which the glutathione ester is protective. Nonetheless, the results obtained so far are consistent with mitochondrial glutathione loss being an important factor in the vulnerability of cells in ischemic and post-ischemic brain.

Implications for neuroprotection

The findings from investigations of the brain in vivo and of cell populations derived from this tissue are consistent with a key role for mitochondrial glutathione in promoting cell viability under some pathological conditions. Additional studies are needed to better understand the normal controls influencing the content of glutathione in brain mitochondria and to further evaluate the possible contribution of mitochondrial glutathione changes in stroke and other brain disorders. Findings to date indicate that treatments to replenish or increase this endogenous antioxidant pool might limit cell death in some brain disorders and could possibly be useful as a prophylactic treatment in situations (such as major surgery) that are associated with an increased risk of brain damage.

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Mitochondrial enzymes and endoplasmic reticulum calcium stores as targets of oxidative stress in neurodegenerative diseases

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ABSTRACT

Considerable evidence indicates that oxidative stress accompanies age-related neurodegenerative diseases. Specific mechanisms by which oxidative stress leads to neurodegeneration are unknown. Two targets of oxidative stress that are known to change in neurodegenerative diseases are the mitochondrial enzyme α -ketoglutarate dehydrogenase complex (KGDHC) and endoplasmic reticulum calcium stores. KGDHC activities are diminished in all common neurodegenerative diseases and the changes are particularly well-documented in Alzheimer's Disease (AD). A second change that occurs in cells from AD patients is an exaggerated endoplasmic reticulum calcium store [i.e., a bombesin-releasable calcium stores (BRCS)]. H_2O_2 , a general oxidant, changes both variables in the same direction as occurs in disease. Other oxidants selectively alter these variables. Various antioxidants were used to help define the critical oxidant species that modifies these responses. All of the antioxidants diminish the oxidant-induced cDCF detectable reactive oxygen species (ROS), but have diverse actions on these cellular processes. For example, α -Keto- β -methyl-n-valeric acid (KMV) diminishes the H_2O_2 effects on BRCS, while trolox and DMSO exaggerate the response. Acute trolox treatment does not alter H_2O_2 -induced changes in KGDHC, whereas chronic treatment with trolox increases KGDHC almost three fold. The results suggest that KGDHC and BRCS provide targets by which oxidative stress may induce neurodegeneration and a useful tool for selecting antioxidants for reversing age-related neurodegeneration.

Introduction

Overwhelming evidence indicates that damage from reactive oxygen species (ROS) occurs in AD brain [Markesbery et al., 1999; Smith et al., 2000]. For example, the reactive aldehyde acrolein is present throughout the AD brain. Many or most tangles contain acrolein, however acrolein is also present in brain areas that do not have tangles suggesting that oxidative stress is more pervasive than tangles [Calingasan et al., 1999]. The presence of hydroxynonenal, another reactive aldehyde [Markesbery et al., 1999], also indicates that reactive aldehydes and lipid damage is extensive in the brain. Protein oxidation [Aksenov et al., 2001; Lyras et al., 1997] and nitration [Smith et al., 1997] indicate damage to protein. ROS-induced damage to all four DNA bases and to RNA (8-hydroxyguanosine) occurs in brains from AD patients [Lyras et al., 1997]. The presence of ferritin, hemoxygenase, and reactive iron are indicators of ongoing oxidative stress [Rottkamp et al., 2001]. A recent study of autopsy brains suggests that measures of oxidative stress are greatest early in the disease and then decline [Nunomura et al., 2001]. Although the inability to make temporal measurements in autopsy brain makes mechanistic approaches equivocal, results in transgenic mice also indicate that oxidative stress is an early change. In these mice, A β is deposited in brain because of over expression of a



human amyloid precursor protein transgene with a double mutation found in a Swedish family with early onset AD. Measures of oxidative stress (isoprostane levels) in these mice precede the surge of A β production and plaque formation by more than a month [Pratico et al., 2001]. Whether a similar, early increase in isoprostanes occurs in human brain is unknown. The specific targets of this oxidative stress and how the changes might be reversed is the focus of this review.

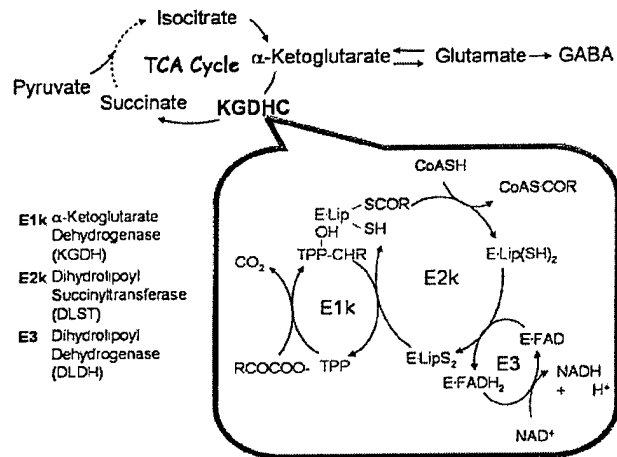
The α -Ketoglutarate Dehydrogenase Enzyme Complex (KGDHC) and neurodegenerative disease.

Brain metabolism is diminished in AD and a possible underlying cause of the decline is a reduction in the activity of the KGDHC. KGDHC consists of three proteins: E1k, E2k and E3. KGDHC is a key and arguably rate-limiting enzyme of the tricarboxylic acid cycle. KGDHC is thiamine-dependent, and is diminished in the brains of individuals with thiamine deficiency (i.e., Wernicke-Korsakoff patients), who have severe memory deficits [Butterworth et al., 1993]. Several groups report that KGDHC is diminished in brains from patients with AD, and no contravening

reports exist [Gibson et al., 2000]. The AD-related reduction in KGDHC activity occurs in genetic and non-genetic forms of AD. Diminished activities occur in brain regions with severe pathology, as well as in areas that show minimal pathology. In the non-genetic forms of the AD, the immunoreactivity of these three components is not altered [Mastrogiovanni et al., 1993], whereas in genetic forms of AD, protein levels of E1k and E2k, but not E3 decline [Gibson et al., 1997]. This suggests that the enzyme can be inactivated by multiple mechanisms. The relation of the decline in KGDHC to the pathophysiology of AD varies with apolipoprotein genotype. In patients with one apolipoprotein E4 allele, the correlation of KGDHC activities to the clinical dementia rating is very high ($r=0.7$). In this same subpopulation, the correlation with plaques ($r=0.11$) and tangles is very low ($r=0.32$) [Gibson et al., 2000]. The decline in KGDHC also occurs in brain regions away from the regions of pathology indicating that the reduction is not just secondary to neurodegeneration. Further, the decline does not appear to be a general loss in mitochondrial function because another mitochondrial enzyme, glutamate dehydrogenase, is unchanged. Thus, brain KGDHC activities decline with AD, and the changes appear to be pathophysiologically important.

KGDHC is diminished in several neurodegenerative disorders. These include Progressive Supranuclear Palsy [Park et al., 2001], Parkinson's Disease [Gibson et al., 2003] and Huntington's Disease [Klivenyi et al., 2004]. As in AD, the decline occurs in both areas of neurodegeneration and in other brain areas. Further, the results are consistent with KGDHC being more sensitive to the disease process (e.g., some

Figure 1. KGDHC and metabolism





undetectable form of ROS) than other common measures of oxidative stress. For example, the decline in cerebellar KGDHC in PSP patients occurs although there is not an increase in malondialdehyde, protein carbonyl formation or protein nitration [Park et al., 2001].

Consequences of diminished KGDHC activities.

Diminishing KGDHC activities has profound consequences on cell and brain function. In cells, inhibition of KGDHC correlates with release of cytochrome C and activation of caspase pathways, and these events precede alterations in the mitochondrial membrane potential [Huang et al., 2003]. In brain slices, inhibition of acetylcholine synthesis is particularly sensitive to KGDHC inhibition [Gibson and Blass, 1976]. Two experimental approaches suggest that diminished KGDHC activities do not induce the neurodegeneration, but predispose to damage by other means. The first approach is thiamine deficiency, which leads to selective neurodegeneration. A thiamine derivative is a cofactor for KGDHC, and a major consequence of thiamine deficiency is a decline in the activity of KGDHC. However, neither the distribution of KGDHC nor the response of KGDHC to neurodegeneration suggests that KGDHC is directly responsible for neuron death. Instead, the results suggest that the decline in KGDHC predisposes to other insults that promote the neuronal death [Sheu et al., 1998]. A second approach to test the consequences of a diminished KGDHC is to use transgenic mice that have reduced levels of the E3 component of KGDHC, and thus diminished KGDHC activities. These mice do not show any pathological changes. However, lesions induced by MPTP and 3-NP are much larger in these mice [Klivenyi et al., 2004].

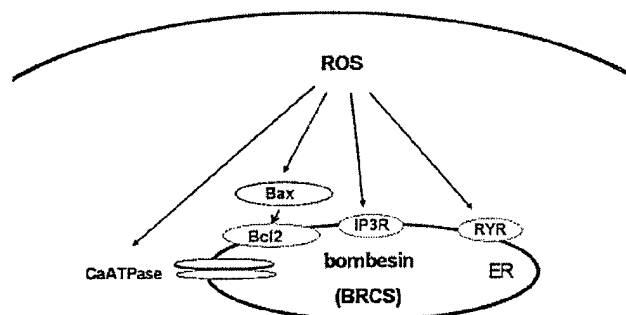
Exaggerated endoplasmic reticulum stores of calcium occur in AD and in animal models of AD.

A second change that accompanies AD is an exaggeration in the internal pools of calcium. Both mitochondrial [Gibson et al., 1997] and endoplasmic reticulum [Ito et al., 1994; Gibson et al., 1996] stores differ between cells from AD patients and controls. The increase in $[Ca^{2+}]_i$ after the addition of bombesin or bradykinin in the absence of calcium is used to assess endoplasmic reticulum stores of calcium.

Experimentally, these are defined as bombesin or bradykinin releasable calcium stores (BRCS). The BRCS are exaggerated in fibroblasts from AD

patients [Ito et al., 1994]. The increases in these pools appear related to the changes in capacitative calcium entry [Leissring et al., 2000; Yoo et al., 2000] and to the reduced flux of calcium into the cells [Peterson et al., 1985]. BRCS has been examined in fibroblasts from multiple individuals with AD including non-genetic forms of AD, and in those bearing PS1 [Ito et al., 1994] and APP [Gibson et al., 1997] mutations. Increases in BRCS also occur in cells that have been transfected with mutant PS-1 [Guo et al., 1996] and in both fibroblasts and neurons from transgenic mice bearing a presenilin-1 mutation [Leissring et

Figure 2. Bombesin releasable calcium stores (BRCS).





al., 2000]. Thus, many approaches indicate that this calcium store is altered in cells from AD patients. The possible actions of ROS on the BRCS are shown in Figure 2.

KGDHC as a target of oxidative stress and for reversal with antioxidants.

Oxidants produce inactivation of KGDHC just as seen in AD. KGDHC is inactivated by a variety of oxidants including peroxynitrite, NO [Park et al., 1999], hydroxynonenal [Humphries et al., 1998], H_2O_2 (in mM concentrations), chloramine (μM concentrations), and sodium hypochlorite (in nM concentrations). H_2O_2 diminishes KGDHC activity in synaptosomes [Chinopoulos et al., 1999], fibroblasts [Gibson et al., 2002], and N2a cells. Although all forms of AD have reduced brain KGDHC activities, protein levels as determined by immunoreactivity decline in some forms of AD, but not others. Even related oxidants can produce a dichotomy similar to that which occurs in AD brain. Both NO and peroxynitrite diminish KGDHC activities. However, peroxynitrite, but not NO, diminishes immunoreactivity of E1k and E2k. The first pattern is similar to that observed in AD patients bearing the APP670/671 mutation (Table I). The second pattern is similar to that seen in AD patients with no known genetic basis.

Table I. Selective changes in KGDHC immunoreactivity with oxidative stress.

	E1k	E2k	E3
NO (SNP)	↔	↔	↔
Peroxyntirite	↓	↓	↔

KGDHC activity is reduced in animal and cell models with increased oxidative stress. Transgenic SOD2 knockout mice have reduced KGDHC activities in their brains [Hinerfeld et al., 2004]. KGDHC is diminished in cells that over-express MAO, and increased substrate (i.e., more ROS) exaggerates the reduction [Kumar et al., 2003].

The sensitivity of KGDHC to ROS suggests that antioxidants should protect KGDHC. The interactions with KGDHC have been studied with H_2O_2 . H_2O_2 produces a dose dependent increase in reactive oxygen species as detected with cDCF. Trolox reduces the ROS production in a dose-dependent manner [Gibson et al., 2002] [Fig. 3]. However, under these same conditions, trolox does not protect KGDHC against H_2O_2 [Fig. 4]. This was also true of the antioxidants N-

Figure 3. H_2O_2 induced increase in ROS is sensitive to Trolox (1 hour treatment)

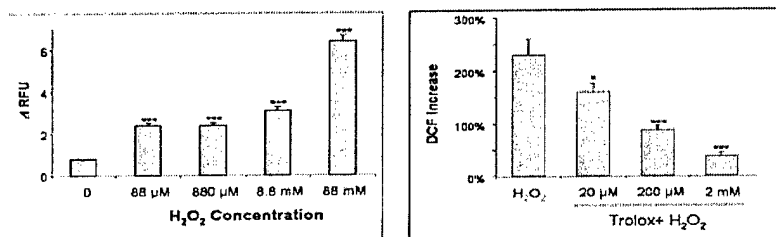
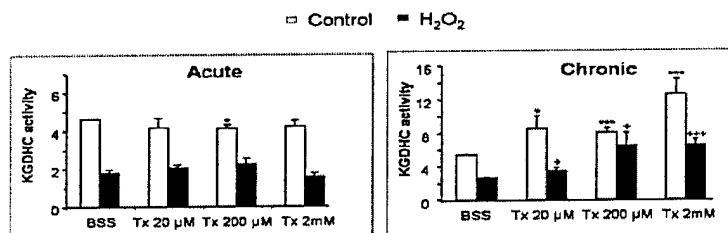


Figure 4. Trolox provides limited protection of KGDHC from H_2O_2





acetylcysteine and DMSO. On the other hand, if KGDHC was incubated with trolox for five days, there was a dose-dependent increase in KGDHC. Following H_2O_2 treatment, all trolox treated cells had higher KGDHC activities than control cells or H_2O_2 alone. The intermediate concentration of trolox diminishes the H_2O_2 induced reduction in KGDHC activities by 50%. [Gibson et al., 2002]. These results suggest that under some conditions with some antioxidants, it is possible to protect KGDHC.

BRCS as a target of oxidative stress and reversal with antioxidants.

BRCS are selectively altered by various oxidants (Table II). BRCS is increased by tert-butylhydroperoxide (t-BHP), H_2O_2 , and SNAP, is unaffected by SIN-1 or SNP, and is diminished by HX/XO. These oxidant produce ROS that can be distinguished with other fluorescent indicators including DAF and amplex red (data not shown).

The interactions of the antioxidant trolox with H_2O_2 -induced changes in BRCS are not predictable [Figure 5. Gibson et al., 2002]. Under the

conditions that reduce H_2O_2 -induced cDCF detectable ROS, trolox exaggerates H_2O_2 -induced increases in BRCS.

However, chronic treatment with trolox has no effect on BRCS (i.e., nearly the opposite effects that are observed with KGDHC). Acute treatment with DMSO also exaggerates the BRCS while NAC has no effect (data not shown). An understanding of the interaction of various oxidant species with various antioxidants will possibly reveal which species lead to the AD-like changes in BRCS.

Further experiments tested the ability of α -keto- β -methyl-n-valeric acid (KMV) to reduce c-DCF detectable ROS and interact with BRCS (Figure 6). KMV appears to be a unique antioxidant. KMV diminishes c-DCF-detectable ROS that are induced by H_2O_2 , hypoxia and SIN-1, but does not neutralize DAF-detectable NO induced by SIN-1. KMV reduces both BRCS and the H_2O_2 -induced change in BRCS. Thus, KMV is better than trolox at protecting against H_2O_2 when BRCS is regarded as the target. The results suggest that the same H_2O_2 -induced ROS that reacts with KMV may also underlie the changes in BRCS related to AD.

Table II. Select changes in BRCS with various oxidants

	DCF-ROS	Change in BRCS
H_2O_2	++	+
SIN-1	+++++	o
t-BHP	+++	++
HX/XO	+++++	-
SNAP	++++	++
SNP	++	o

Figure 5. Trolox exaggerates the effects of H_2O_2 on BRCS

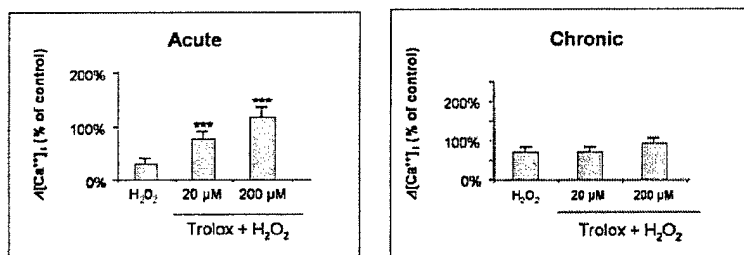
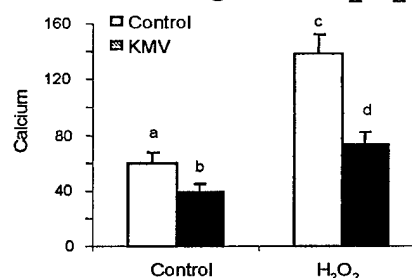


Figure 6. KMV protects BRCS against H_2O_2





Conclusions

Oxidative stress is a common feature of neurodegenerative diseases. This may lead to oxidation of key components of BRCS and of KGDHC. The changes in these processes in disease can be patterned by select oxidants. The results suggest these changes may be important in normal signaling of the molecules as well in neurodegeneration. Therefore, reversal of the changes in these processes by select antioxidants may be beneficial.

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Mitochondrial Nitric-Oxide Synthase: Enzyme expression, characterization and regulation

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ABSTRACT

Nitric oxide is generated *in vivo* by nitric-oxide synthase (NOS) during the conversion of L-Arg to citrulline. Using a variety of biological systems and approaches emerging evidence has been accumulated for the occurrence of a mitochondrial NOS (mtNOS), identified as the alpha isoform of neuronal or NOS-1. Under physiological conditions, the production of nitric oxide by mitochondria has an important implication for the maintenance of the cellular metabolism, i.e. modulates the oxygen consumption of the organelles through the competitive (with oxygen) and reversible inhibition of cytochrome c oxidase. The transient inhibition suits the continuously changing energy and oxygen requirements of the tissue; it is a short-term regulation with profound pathophysiological consequences. This review describes the identification of mtNOS and the role of posttranslational modifications on mtNOS' activity and regulation.

1. INTRODUCTION

Nitric oxide is formed from L-arginine by nitric-oxide synthase (NOS), which oxidizes the guanidino nitrogen of arginine, releasing nitric oxide and citrulline (Bredt and Snyder, 1990; 1994). Three main NOS are expressed in mammals and differ in their functions, amino acid sequence, post-translational modification, and cellular location. Two NOS, neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3), are constitutively expressed and involved in signal cascades (Mayer et al., 1989; 1990; Bredt and Snyder, 1990). The third NOS is cytokine-inducible (iNOS or NOS-2) and functions as both a regulator and effector of the immune response (Stuehr and Marletta, 1987; Förstermann et al., 1992). The consequence to this diversity of location and function is a specific regulation of each isoform. For example, NOSs differ significantly regarding Ca²⁺ levels required to bind calmodulin, which triggers heme reduction and nitric oxide synthesis (Abu-Soud et al., 1994; Panda et al., 2001). They also have different capacities to be up- or down regulated by Ser/Thr phosphorylation (Fulton et al., 1999; Harris et al., 2001). Using rat liver, we provided unequivocal evidence for a localization of a NOS (mitochondrial NOS or mtNOS) at the inner membrane of mitochondria (Giulivi et al., 1998; Tatoyan and Giulivi, 1998). Given NOS' diverse biochemical characteristics, it could be postulated that the production of nitric oxide by mitochondria is highly regulated because of the critical role that this molecule has on cellular respiration (Brown and Cooper, 1994; Cleeter et al., 1994; Poderoso et al., 1996). In the following sections, the identification of mtNOS and the role of posttranslational modifications on its activity and regulation are summarized and discussed.

2. PRODUCTION OF NITRIC OXIDE BY MITOCHONDRIA

Our studies were the first providing evidence for production of nitric oxide by purified mitochondria (Giulivi et al., 1998; Tatoyan and Giulivi, 1998; Giulivi, 1998; Giulivi et al., 1999). This production of nitric oxide was demonstrated by using direct (L-citrulline production, evaluated by using colorimetric or radiolabeled compounds) and indirect techniques (nitric oxide generation, evaluated by electron paramagnetic resonance with spin trapping; nitric oxide-dependent oxidation



of oxymyoglobin). More evidence was furnished by the measurement of NOS activity in mitochondria isolated from purified hepatocytes, (abrogating the putative contamination of Kupffer cells), and mitoplasts (mitochondria stripped of the outer membrane). Isolation and purification of mtNOS, mainly localized at mitochondrial membranes, allowed obtaining critical kinetic constants and dependence on cofactors and cosubstrates (Tatoyan and Giulivi, 1998; Giulivi, 2003).

Other labs had reached similar conclusions or extended the knowledge in this field in terms of the occurrence of a mtNOS by using less purified mitochondrial fractions, isolated cells, and various approaches such as colocalization of mitochondria with a production of nitric oxide in intact cells, nitric oxide detection by electrode in wild-type and nNOS KO mice mitochondria, among others (Bates et al., 1995, 1996; Kobzik et al., 1995; Ghafourifar and Richter, 1997; López-Figueroa et al., 2000; Kanai et al., 2001; Dedkova et al., 2004).

Considering that the constitutive forms of nitric-oxide synthase, i.e., nNOS and eNOS, account for the rapid, transient, calcium-dependent production of nitric oxide (Mayer et al., 1989; 1990; Bredt and Snyder, 1990), thus, it would be expected that increases in mitochondrial calcium be required to activate mtNOS. In line with this assumption, stimulation of nitric oxide production by mtNOS was observed by bolus additions of calcium to mitochondria (Ghafourifar and Richter, 1997). The authors proposed that uptake of calcium by respiring mitochondria may lead to increased peroxynitrite formation in mitochondria, which in turn causes calcium release (Schweizer and Richter, 1996) via the pyridine nucleotide-dependent pathway (Lötscher et al., 1979) followed by mtNOS deactivation. These observations have been interpreted as part of a feedback loop, which prevents calcium overloading and allows its release preserving membrane potential (Ghafourifar and Richter, 1997).

An apparent discrepancy rises in terms of the role of calcium on the rate of oxygen consumption by mitochondria: on one hand, mitochondrial calcium increases the rate of oxygen consumption as a result of the activation of calcium-activated dehydrogenases (Hansford, 1985; Denton and McCormack, 1985; 1993; McCormack et al., 1990), and on the other, by activating mitochondrial nitric-oxide synthase, decreases the oxygen consumption by inhibiting cytochrome oxidase activity. When we evaluated the rates of State 3 oxygen consumption in the presence of Ng monomethyl-L-Arg (NMMA), a competitive inhibitor of mtNOS, at various concentrations of calcium, the $K_{0.5}$ was 0.1 μM (similar to that required for the activation of the Krebs' cycle) whereas in those with L-Arg (in which mtNOS was saturated with L-Arg) the $K_{0.5}$ was 0.45 μM (Traaseth et al., 2004). By plotting the difference between the rates of oxygen consumption in State 3 with L-Arg and with NMMA at various calcium concentrations, a $K_{0.5}$ of 0.3 μM was obtained, similar to the $K_{0.5}$ (0.26 μM) of the dependence of the rate of nitric oxide production on calcium concentrations, and within the values of other $K_{0.5}$ found for purified nNOS (Mayer et al., 1989; Bredt and Snyder, 1992). Thus, the difference between these $K_{0.5}$ indicates that the activation of dehydrogenases, followed by the activation of mtNOS would result in the modulation of the Krebs' cycle activity by the modulation of nitric oxide on the respiratory rates (Traaseth et al., 2004). This would ensue in changes in the NADH/NAD⁺ and ATP/ADP ratios, which would influence the rate of the cycle and the oxygen diffusion.

2. BIOCHEMISTRY OF MITOCHONDRIAL NITRIC-OXIDE SYNTHASE

The identification of mtNOS was a critical step in this research because it allowed the assignment of this protein to one of the known isoforms, or to depict it as a novel isoform. By using nitric-oxide electrodes to follow the production of nitric-oxide by mitochondria, mtNOS has been identified as the nNOS, likely to be coded by the same gene as nNOS for nNOS K.O. mice have no mtNOS (Kanai et al., 2001). Independently, our lab reached the same conclusions in terms of identifying mtNOS as the alpha isoform of nNOS and expanded this concept to identify the isoform and posttranslational modifications (Elfering et al., 2002). Briefly, purified mtNOS was separated by 2D-electrophoresis, followed by in-gel digestion with either trypsin or endoproteinase V8, and



MALDI-ToF analyses were performed on the eluted fragments. The resulting sequences were blasted against in-silico trypsin- or V8 digested proteins from the PDB and matched to sequences of constitutive rat nNOS (Elfering et al., 2002). Given that mouse bNOS has 5 isoforms (known as bNOS-1, bNOS2, bNOS-beta, bNOS-gamma, and bNOS-MU or muscle-specific; Ogura et al., 1993; Silvagno et al., 1996; Brenman et al., 1997) produced by alternative splicing of mRNA, the question remained whether mtNOS was one of these products or represented a novel alternative splicing product. Some of the fragments obtained with MALDI excluded NOS-gamma and NOS-2, NOS-beta and NOS-gamma seemed unlikely candidates based on their MW, suggesting that either NOS-1 or -MU was mtNOS. RT-PCR experiments performed on enriched poly(A)⁺ mRNA from rat liver (using primers based on MALDI-ToF sequences or gene-specific) and PCR experiments performed on rat liver cDNA resulted in the amplification of segments of the transcript corresponding to nNOS alpha isoform.

Our results combined from MALDI, MW, and pI indicated that mtNOS is bNOS, excluding the possibility of a novel isoform or an alternative splicing product. The identification of this enzyme was crucial because it will allow studying its biochemistry in detail with the previous knowledge that we have on the bNOS isoform.

Other studies had emerged perceiving mtNOS as a novel or other than the nNOS isoform as indicated above (Brookes, 2004 and references therein). Distinction needs to be made on what is considered identification parameters for a protein in biochemistry. Protein characterization (not identification) based on western blotting technique is based on the crossreactivity of an antibody with a small segment of the protein (or epitope), which is usually smaller than the actual antigen used for immunization (usually 1% to 15% of the protein). Thus, a positive result in a western blot should be understood as the crossreactivity of an antibody with a certain epitope in the protein, not necessarily indicating 100% homology with the rest of the protein. Considering that proteins like eNOS, iNOS, and nNOS have a 49 to 56% homology and that most of the commercially available antibodies are directed to the C-terminal half of the enzyme (or reductase domain) which shows pronounced sequence similarities to cytochrome P450 reductase (Bredt et al., 1991) and where most of the homology among NOSs is present (48 to 55%) provides limited evidence for identification purposes. Even the combination of western blotting with MW calculated from SDS-PAGE (where the error associated with proteins with a MW higher than 100 kDa is expected to be higher than 10% because of the limited availability of high MW standards and the lack of linearity between MW and mobility in this range, aside from the error constituted by the presence of posttranslational modifications such as acylation or glycosylation; Weber and Osborn, 1969; Laemmli, 1970) furnish inadequate information for identification purposes.

3. POSTTRANSLATIONAL MODIFICATIONS OF mtNOS AND THEIR ROLE IN NITRIC OXIDE REGULATION

3.1. Acylation Pattern of Mitochondrial Nitric-Oxide Synthase

All three NOSs (i.e., b-, e-, and mac-NOSs) were recovered from primary cells in both a soluble and a particulate fraction (Pollock et al., 1991; Liu and Sessa, 1994; Hecker et al., 1994). Endothelial cNOS, which was recovered from resting cells almost exclusively in the latter fraction (Pollock et al., 1991), was labeled when host cells were incubated with radioactive myristic acid (Liu and Sessa, 1994). Here, the amino-terminal group was co-translationally linked to myristic acid, supported by the following evidences: 1) the inhibition of myristic acid incorporation by a mutation of the amino-terminal Gly indicating that the fatty acid was bound by an amide bond to the protein; 2) cycloheximide treatment abolished the incorporation of myristic acid indicating that the fatty acid was incorporated during protein synthesis; and 3) the identification of myristic acid was performed by the release of myristoyl methyl ester from the protein, after acid methanolysis followed by hydroxylamine treatment. Later, it was reported that eNOS is also palmitoylated at the Cys



residues. These acylations allowed the detection of the enzyme close to calveolin, located at the plasma membrane, in intact endothelial cells (Busconi and Michel, 1993). With bNOS isolated from rat but not rabbit, most of the protein sedimented in a subcellular fraction whose marker enzymes were typical of endoplasmic reticulum (Liu and Sessa, 1994).

mtNOS was found mainly localized at the inner mitochondrial membrane, requiring the presence of CHAPS to solubilize the enzyme from mitochondrial membranes, indicating that the enzyme is tightly bound to the membrane. Experiments designed to investigate the putative acylation of mtNOS resulted in the finding that myristic acid is linked to mtNOS through an oxy- or thio-ester bond. Myristic acid was probably bound during a reversible, posttranslational process, catalyzed by acyltransferases. It should be noted that the pattern of acylation found with mtNOS differs from that observed with eNOS, in which an N-terminal myristoylation and palmitoylation of Cys residues were found. No data on acylation are available for the other two NOS, i.e., bNOS and macNOS; however, their main soluble localization may indicate a low or negligible acylation.

The occurrence of lipid-protein linkages in mtNOS may indicate an alternative modulatory role based on acylation-deacylation processes. The exact function of acylating proteins is still not known, and in this context, protein-protein interactions, membrane localization, or subcellular distribution has been proposed. In this case, it could be speculated that acylation of mtNOS is implicated in the regulation of mitochondrial nitric oxide production. If acylated mtNOS could be incorporated to the mitochondrial membranes, this localization might be advantageous for the following reasons: first, nitric oxide will be produced closer to the target site, cytochrome oxidase, thus minimizing secondary reactions; second, it will extend the lifetime of nitric oxide considering that this molecule when is produced in an aqueous, aerated solvent is consumed faster than if it is produced close to or in the membrane because nitrosyl dioxy --the product of nitric oxide and oxygen-- can be stabilized by H bonds in an aqueous milieu (Beckman, 1996); and third, it may facilitate the targeting of the protein to mitochondria given that hydrophobic substrates tend to concentrate in these organelles.

Of note, proteins modified by ester bonds are potentially subject to dynamic regulation: the linkage and cleavage of palmitic acid to proteins are catalyzed by yet uncharacterized palmitoylthiotransferases and palmitoylthioesterase (Resh, 1994). These processes may underlie the ability of the enzyme, as with eNOS, to form stable, but dynamically regulated, associations with cell membranes (Busconi and Michel, 1993). This type of regulation could be also present in mitochondria and added a type of regulation to enzymatic activity by controlling the compartment in which the enzyme is present.

3.2. Phosphorylation of mtNOS

In response to agonists, eNOS accumulated phosphate and became soluble (Michel and Busconi, 1993) probably by decreasing the positive charge of a region that contributed electrostatically to the bind of eNOS to lipid. Other studies provided evidence that all three NOS isoforms immunoprecipitated from host cells are phosphorylated (Michel and Busconi, 1993; Dawson et al., 1993). It has been shown that kinase- and phosphatase-dependent events occurring in cells modified NOSs activity (Bredt et al., 1992; Nakane et al., 1991; Brune and Lapetina, 1991). Recently, it has been reported that regulation of eNOS activity involves phosphorylation (Chen et al., 1999; Dimmeler et al., 1999) and coordinated signaling through Ser-1177 and Thr-495 by multiple protein kinases and phosphatases (Michell et al., 2001).

Previous studies performed by our lab indicated that a phosphorylation was present in mtNOS at the fragment comprised between amino acids 1408 and 1421. Interestingly, Ser-1177 or 1179 from human or bovine eNOSs, respectively, which was found to play a critical role in the coordinated phosphorylation/dephosphorylation of the protein (Michell et al., 2001), is homologous to Ser-1413 in rat bNOS, suggesting that this position could be subjected to phosphorylation. It is tempting to hypothesize that if ATP and respiratory substrate levels are high, then phosphorylation



of mtNOS may enhance NOS' activity (by analogy with eNOS). This regulation will increase the production of nitric oxide, thus allowing an inhibition of cytochrome oxidase and the consequent production of ATP. This pathway will allow that oxygen and other substrates get to cells that not necessarily are close to blood vessels, assuring a homogenous distribution.

4. PHYSIOLOGICAL ROLE OF mtNOS

We demonstrated that, under physiological conditions, the production of nitric oxide by mitochondria has an important implication for the maintenance of the cellular metabolism, i. e., nitric oxide (produced by rat liver mitochondria) modulated the oxygen consumption of the organelles (Giulivi, 1998; 2003; Giulivi et al., 1999). This effect was achieved through the reversible inhibition of cytochrome oxidase by nitric oxide (Giulivi, 2003; Haynes et al., 2003 and references therein). This transient inhibition suits the continuously changing energy and oxygen requirements of the tissue. However, if a sustained inhibition of cytochrome c oxidase is allowed, then other deleterious effects may happen: inhibition of ATP synthesis, release of cytochrome c (Ghafourifar et al., 1999), increased oxygen radical production (Sarkela et al., 2001) and nitration of critical biomolecules (Aulak et al., 2001; Elfering et al., 2003; Traaseth et al., 2004).

Several lines of evidence indicated that the gaseous molecule nitric oxide by binding to the heme moiety of soluble guanylate cyclase leads to its activation, and the formation of cGMP triggers a variety of events in various organs (Garthwaite and Garthwaite, 1987; Marletta, 1989; Moncada et al., 1991; Dawson et al., 1992). However, our research and that of others had indicated cytochrome oxidase as a different target for nitric oxide, by which mediates other processes not mediated or triggered by cGMP. Our hypothesis is that nitric oxide produced by mitochondria has a short-term, regulatory role on energy metabolism, oxygen consumption, and the inherent free radical production. The broader implications of the present work can help to redefine the way we view regulation of oxygen consumption *in vivo*. Based on our initial findings, it has been proposed that mitochondrial production of nitric oxide helps average oxygen utilization between cells at different distances from capillaries. The basic concept is that nitric oxide will slow oxygen consumption by cells closest to blood vessels, allowing oxygen to penetrate to cells at the boundary of becoming hypoxic. In addition, nitric oxide would help dilate blood vessels and potentially increase oxygen delivery to borderline hypoxic cells (Giulivi, 2003; Haynes et al., 2003). Indeed, by following a similar line of thought, a mechanism for fireflight flashing has been proposed in which the role of nitric oxide is to transiently inhibit mitochondrial respiration in photocytes and thereby increasing the availability and level of oxygen in the peroxisomes, oxygen being the species considered as the biochemical trigger for light production (Trimmer et al., 2001).

This emerging field in mtNOS is important, as it will expand the mechanisms by which cells consume oxygen and how changes in pO_2 are coped on a short-time framework. Studies in this field will provide key information on the molecular mechanisms of cellular respiration, and will likely lead to the design of better therapies to prevent pathological ischemic events during such diseases such as heart or brain stroke, and to advance our knowledge in the field of mitochondrial diseases.

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Protection Against Ischemic Brain Injury by Inhibition of Mitochondrial Oxidative Stress

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Abstract

Mitochondria are both targets and sources of oxidative stress. This dual relationship is particularly relevant in experimental paradigms modeling ischemic brain injury. One mitochondrial metabolic enzyme that is particularly sensitive to oxidative inactivation is pyruvate dehydrogenase. This reaction is extremely important in the adult CNS that relies very heavily on carbohydrate metabolism as it represents the sole bridge between anaerobic and aerobic metabolism. Oxidative injury to this enzyme and to other metabolic enzymes proximal to the electron transport chain may be responsible for the oxidized shift in cellular redox state that is observed during approximately the first hour of cerebral reperfusion. In addition to impairing cerebral energy metabolism, oxidative stress is a potent activator of apoptosis. The mechanisms responsible for this activation are poorly understood but likely involve the expression of p53 and possibly direct effects of reactive oxygen species on mitochondrial membrane proteins and lipids. Mitochondria also normally generate reactive oxygen species and contribute significantly to the elevated net production of these destructive agents during reperfusion. Approaches to inhibiting pathologic mitochondrial generation of reactive oxygen species include mild uncoupling, pharmacologic inhibition of the membrane permeability transition, and simply lowering the concentration of inspired oxygen. Anti-death mitochondrial proteins of the Bcl-2 family also confer cellular resistance to oxidative stress, paradoxically through stimulation of mitochondrial free radical generation and secondary upregulation of antioxidant gene expression.

Mitochondrial Targets of Oxidative Stress

Several lines of evidence indicate that oxidative stress is a primary mediator of neurologic injury following cerebral ischemia. The extent of delayed neuronal death correlates well with pre-lethal markers of oxidative molecular alterations. Neuroprotection is observed following the use of antioxidants and inhibitors of free radical producing enzymes, e.g., nitric oxide synthetase. In addition, neuroprotection is evident in genetic animal models where genes coding for enzymes that promote oxidative stress are knocked down or out, and where genes coding for antioxidant enzymes, e.g., superoxide dismutase (SOD) are overexpressed (see (Lewen et al, 2000) for review).

Virtually every cellular and extracellular molecular component is potentially sensitive to damage caused by oxidative stress. Oxidative modification to DNA, RNA, proteins, lipids, and small metabolites occur during ischemia/reperfusion. Our research focuses on the mitochondrion and its components as both targets and mediators of oxidative reperfusion injury (Fiskum et al, 1999; Murphy et al, 1999). From both *in vitro* studies with neural cells (Myers et al, 1995) and animal models of global cerebral ischemia (Liu et al, 1998), we conclude that mitochondrial energy metabolism is extremely sensitive to impairment by reactive oxygen and nitrogen species and that mitochondrial oxidative stress limits metabolic recovery and promotes the intrinsic pathway of apoptosis.



One hypothesis we are testing is that during reperfusion, pyruvate dehydrogenase (PDH) is oxidatively modified and inactivated (Rosenthal et al, 1992; Bogaert et al, 2000), resulting in impaired oxidative energy metabolism and exacerbation of post-ischemic brain lactic acidosis. PDH enzyme activity is lost when purified enzyme is exposed to systems that generate hydroxyl radicals (Bogaert et al, 2000) or peroxynitrite (E. Martin, unpublished), two reactive oxygen/nitrogen species strongly implicated in reperfusion brain injury. In addition to inactivation of PDH, the activity of the electron transport chain Complex I (NADH-CoQ oxidoreductase) is also depressed during reperfusion (Almeida et al, 1995), which could be particularly important since this Complex is normally the rate-limiting step of the electron transport chain (Davey et al, 1997). The relative importance of damage to components of the electron transport chain compared to upstream metabolic enzymes, e.g., PDH, is at this juncture unknown. However, decreased production of NADH by PDH and TCA cycle dehydrogenases may be responsible for the hyper-oxidized redox state of NAD(H) and components of the mitochondrial electron transport chain that occurs during the first hour of reperfusion after global cerebral ischemia (Rosenthal et al, 1995). If the electron transport chain was metabolically rate-limiting during reperfusion, the NAD(H) redox state should be relatively reduced rather than oxidized. Postischemic oxidation of NAD(H) may therefore constitute an important clue for the identification of the most important metabolic targets of reperfusion injury.

Other possible explanations for the effect of ischemia/reperfusion on NAD(H) redox state include depletion of pyridine nucleotides through PARP activation (Wang et al, 2002), and release of mitochondrial NAD(H) and NADP(H) from the mitochondrial matrix into the cytosol, e.g., what occurs following activation of the mitochondrial membrane permeability transition (MPT) (Chinopoulos et al, 2003). In addition to inhibiting PDH and other mitochondrial enzyme activities, ROS are potent activators of both PARP and the MPT (Prabhakaran et al, 2004; Kowaltowski et al, 1999). Moreover, the metabolism of H_2O_2 and other peroxides via the glutathione peroxidase/reductase system can contribute to the oxidative shift in pyridine nucleotide redox state. Irrespective of the mechanism by which cerebral reperfusion causes this shift in redox state, the associated decrease in reducing power could limit detoxification of peroxides and maintenance of reduced protein sulfhydryl groups, thereby contributing to the prolonged oxidative stress characteristic of reperfusion tissue injury.

While oxidative damage to cerebral energy metabolism is a critical contributor to delayed, necrotic neuronal death, oxidative stress is also a powerful initiator of apoptosis, which also contributes significantly to ischemic neural cell death (Hou and MacManus, 2002; DeGracia et al, 2002). The mechanism by which oxidative stress promotes apoptosis is far from understood. Possible mechanisms include increased expression of p53, a redox-sensitive transcriptional activator of several pro-apoptotic genes that also directly induces release of mitochondrial cytochrome c (CytC) through its interaction with the anti-apoptotic mitochondrial protein Bcl-X_L (Miller et al, 2000; Soengas et al, 1999; Chipuk et al, 2003). Reactive oxygen and nitrogen species can also induce the release of CytC from mitochondria through promotion of the MPT (Kowaltowski et al, 1999; Borutaite et al, 1999), although this event is more likely to cause necrosis due to the devastating effects of the MPT on mitochondrial energy metabolism. Oxidative alterations to mitochondrial membrane lipids or apoptotic proteins might also promote the release of CytC and other pro-apoptotic mitochondrial proteins through both MPT- and non-MPT-dependent mechanisms.

Neuroprotection by Avoiding Hyperoxia During Cerebral Reperfusion

Intracellular conditions that exist early during reperfusion, e.g., low pH and high $[Ca^{2+}]$, can promote the generation of ROS by mitochondria and other sources (Fiskum, 1997). Microdialysis measurements demonstrate high levels of hydroxyl radical production during the first 30 – 45 min of reperfusion (Piantadosi and Zhang, 1996). During this same period, hyperoxia exacerbates the



oxidized shift in mitochondrial redox state and delays recovery of evoked potentials compared to what is observed with normoxic animals (Feng et al, 1998). Several other studies have compared hyperoxic to normoxic reperfusion using histopathology as the outcome measure. Halsey implanted O_2 electrodes in the brains of rats before subjecting them to a 20 min global ischemic insult and found a positive correlation between reoxygenation level and severity of neuronal damage (Halsey, Jr. et al, 1991). Gerbils treated with 100% O_2 after 15 min bilateral carotid occlusion sustained increased white matter damage (Mickel et al, 1990). A study using 15 min of cardiac arrest in dogs followed by hyperoxic resuscitation found a significant increase in the total number of injured neurons in the brain stem and spinal cord within one hour of resuscitation (Marsala et al, 1992). Our preliminary results using a 10 min canine cardiac arrest model and stereologic cell counting indicate a significant reduction in hippocampal neuronal death using normoxic compared to hyperoxic resuscitation (V. Vereczki, unpublished). In contrast, Agardh used a rat model of transient global ischemia and failed to demonstrate differences in 7 day neuronal damage after resuscitation with 100% O_2 compared to normoxia or hypoxia (Agardh et al, 1991). Lipinski et al. also found no difference in hippocampal neuronal death 72 hr following cardiac arrest in rats ventilated on 100% or 21% O_2 (Lipinski et al, 1999). This model is, however, significantly different from the canine cardiac arrest model or from most human cardiac arrest scenarios as the animals experience severe hypoxia prior to cardiac arrest.

The few reported comparisons of neurologic outcome following hyperoxic and normoxic reperfusion strongly suggest that hyperoxic resuscitation is detrimental. Using a 9 min canine cardiac arrest model, Zwemer found that resuscitation with 100% inspired O_2 resulted in worsened 12 and 24 hr neurologic outcome when compared to animals receiving 21% O_2 (Zwemer et al, 1994). This difference was eliminated when animals were pretreated with an antioxidant prior to the arrest and hyperoxic resuscitation. In our canine experiments using 10 min cardiac arrest, neurologic impairment measured at 24 hr was significantly worse in animals ventilated on 100% O_2 during and for 1 hr after resuscitation than that exhibited by dogs resuscitated on 21% O_2 and subsequently ventilated on 21 – 30% O_2 to maintain normal PaO_2 (Liu, Rosenthal, Haywood, Miljkovic-Lolic, Vanderhoek, and Fiskum, 1998). The one published negative study is the Lipinski report where no difference in 72 hr neurologic impairment was observed following asphyxia-induced cardiac arrest in rats (Lipinski, Hicks, and Callaway, 1999). The only long-term outcome study focused on mortality and used the gerbil bilateral carotid occlusion model. Mickel and colleagues found that animals exposed to 100% O_2 for 3-6 hr after 15 min global cerebral ischemia experienced a 3-fold increase in 14 day mortality compared with those allowed to breathe room air after ischemia (Mickel et al, 1987).

Mitochondria as Sources of Reactive Oxygen Species

Superoxide is a normal byproduct of mitochondrial respiration and accounts for 1-2% of O_2 consumed by mitochondria. Because of its extremely high reactivity and short half-life, it normally dismutates to H_2O_2 either spontaneously or via catalysis by mitochondrial or cytosolic superoxide dismutases. While the metabolism of H_2O_2 via peroxidases can, under some circumstances, lead to oxidative stress due to an oxidized shift in cellular redox state, the primary toxicity of elevated superoxide and H_2O_2 production is exerted by other metabolites. These products include the hydroxyl radical, generated by metal catalyzed reduction of H_2O_2 , and peroxynitrite, generated by

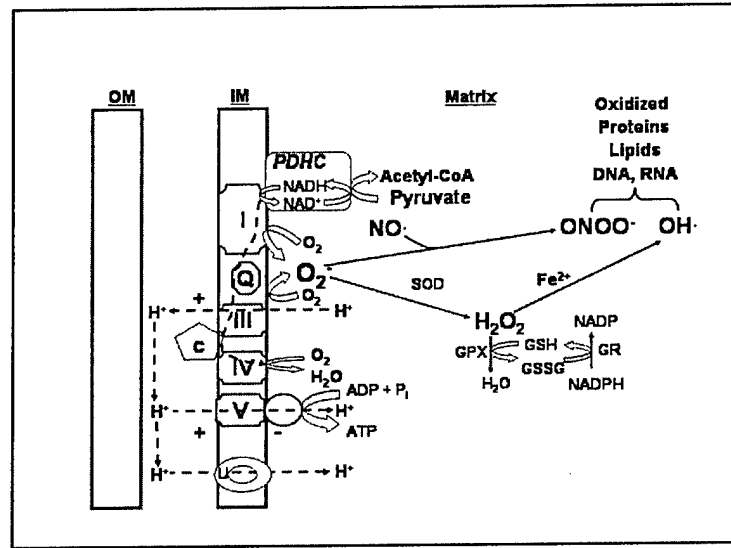


Fig. 1. Mitochondrial Oxidative Stress. Redox sites associated with Complex I and III of the electron transport chain generate superoxide, which is either detoxified through superoxide dismutase (SOD) and glutathione peroxidase and reductase (GPX; GR), or converted into toxic hydroxyl radical and peroxynitrite. One target of these metabolites is the pyruvate dehydrogenase complex (PDHC), which catalyzes an extremely important reaction in cerebral energy metabolism

the reaction of superoxide with nitric oxide. Both of these reactive agents are capable of oxidatively modifying proteins, lipids, RNA, and DNA. As there are no known enzymatic systems for

detoxifying either hydroxyl radical or peroxynitrite, endogenous interventions are limited primarily to those that reduce the production of superoxide or nitric oxide, or that promote the non-toxic metabolism of H_2O_2 to H_2O via peroxidase activities. A number of additional exogenous anti-oxidant approaches are available, including the use of iron chelators, spin-traps, and other natural and artificial anti-oxidant compounds.

While the role of mitochondrial ROS production in ischemia/reperfusion injury is often touted as important, little direct evidence is available from *in vivo* experiments. Existing evidence is based on the effects of mitochondrial respiratory inhibitors or uncouplers on markers of oxidative injury. Additional support for a critical role of mitochondrial oxidative stress in acute neuronal cell death comes from *in vitro* experiments using cultured neurons and other neural cell lines exposed to hypoxia and glucose deprivation, or to toxic levels of excitatory neurotransmitters or their agonists. From these and more recent studies, it appears that initial entry of Ca^{2+} through glutamate receptors is accumulated into mitochondria, causing an increase in mitochondrial ROS production that then causes a secondary irreversible entry of Ca^{2+} through redox-sensitive transient receptor potential (Trp) channels (Aarts et al, 2003).

One controversial topic in this field is the involvement of the MPT in Ca^{2+} -induced mitochondrial ROS production. MPT-mediated release of CytC can certainly stimulate mitochondrial



generation of ROS by causing a reduced shift in mitochondrial redox sites associated with superoxide production. The MPT also causes a drop in mitochondrial membrane potential ($\Delta\Psi$) and a loss of mitochondrial pyridine nucleotides, both of which should depress mitochondrial generation of ROS. Recent work suggests, however, that even if mitochondrial NAD(H) were released into the cytosol in response to the MPT, the residual concentration in the mitochondrial matrix in equilibrium with the cytosolic pool could be sufficient to support substantial ROS production (Batandier et al., 2004). The use of MPT inhibitors like cyclosporin A as neuroprotectants both *in vivo* and *in vitro* has met with mixed success. Increasing evidence indicates that cyclosporin A is ineffective at blocking the MPT under many conditions and in certain cell types, including neurons (Chinopoulos et al., 2003). Development of more broadly effective MPT inhibitors is therefore needed. One agent that exhibits superior inhibition of the MPT in brain mitochondria is 2-aminoethoxydiphenylborate (2-APB) (Chinopoulos et al., 2003). This drug also inhibits capacitative Ca^{2+} entry associated with Trp channels and may therefore provide a multipotent approach to neuroprotection (Tozzi et al., 2003).

Several mechanism other than, or in addition to the MPT could stimulate mitochondrial ROS production during ischemia/reperfusion. Thermodynamically, any inhibition of electron flow distal to redox sites of superoxide production would promote these reactions. Thus, inhibition of electron transfer in Complex I distal to the putative iron sulfur site of superoxide generation, as occurs with rotenone, greatly stimulates ROS production with NADH-linked respiratory substrates. Inhibition at the distal points in the electron chain, as occurs with nitric oxide at Complex IV, stimulates ROS production at both Complex I and at the Coenzyme Q/Complex III redox site. A similar situation occurs when CytC is released during apoptosis via Bax-mediated formation of pores in the outer membrane (Starkov and Fiskum, 2003). When electron flow is only partially inhibited, the redox-mediated stimulation of ROS production can be counteracted by mild uncoupling, either with exogenous uncoupling agents, e.g., FCCP, or via increased expression or activity of mitochondrial uncoupling proteins. Mitochondrial ROS production is extremely sensitive to inhibition by slight depolarization and oxidized shift in redox state at high membrane potentials (Starkov and Fiskum, 2003). Thus a drop in $\Delta\Psi$ of only 15 mV reduces the rate of NADH-linked, substrate-dependent ROS formation by 50% with little effect on ATP production. Mild uncoupling may therefore constitute an effective means of immediately reducing oxidative stress in acute CNS injury paradigms (Kim-Han et al, 2001; Ferranti et al, 2003).

In addition to inhibiting mitochondrial superoxide production, the net production of ROS can also be reduced by promoting its detoxification to H_2O_2 and then to H_2O . Bcl-2, an anti-death protein normally thought to act by binding to outer membrane pore-forming pro-apoptotic proteins, e.g., Bax, also exhibits an indirect antioxidant activity that is apparent even at the mitochondrial level (Hockenbery et al, 1993). We demonstrated that the inhibition of pro-oxidant-induced MPT by Bcl-2 overexpression is due to increased resistance of pyridine nucleotides to oxidation rather than a direct effect on MPT proteins (Hockenbery, Oltvai, Yin, Millman, and Korsmeyer, 1993). In an attempt to explain this phenomenon, we explored the effects of Bcl-2 and other antiapoptotic Bcl-2 family members on mitochondrial bioenergetics. Through careful calibration techniques, we found that mitochondria from Bcl-2 overexpressing cells do not exhibit higher membrane potential, in contrast to previous reports indicating a difference in $\Delta\Psi$. In agreement with other investigators, we found that overexpression of Bcl-2 was associated with an increase in basal mitochondrial ROS (H_2O_2) production. This counterintuitive phenotype was also observed in cells overexpressing Bcl-X_L and Mcl-1, two additional cytoprotective Bcl-2 family members (A. Kowaltowski, unpublished). Most importantly, when the overexpressing cells were treated for 48 hr with low levels of uncoupler that eliminate the elevated level of mitochondrial ROS production, the cells lose their abnormally high peroxidase activity and their resistance to acute necrotic cell death caused by exposure to high



concentrations of exogenous H_2O_2 . It therefore appears that the anti-oxidant activity of at least 3 antiapoptotic Bcl-2 family members is similar to preconditioning paradigms where sublethal levels of stress cause up-regulation of proteins that protect against normally lethal levels of stressful stimuli. While overexpression of Bcl-2 increases basal ROS production, this effect stimulates the expression of one or more antioxidant enzymes resulting in a net resistance to oxidative stress.

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MITOCHONRIAL UNCOUPLING AS A THERAPEUTIC TARGET FOLLOWING NEURONAL INJURY

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Central Nervous System (CNS) trauma results in several pathophysiological events that contribute to neuronal damage and death, including glutamate-mediated excitotoxicity and the formation of reactive oxygen species (ROS) (Faden et al., 1989; Braughler and Hall, 1992; Azbill et al., 1997; Sullivan et al., 1998; Sullivan et al., 1999a). The ensuing loss of neuronal tissue is believed to evolve in a biphasic manner consisting of the primary mechanical insult and a progressive secondary necrosis (Cooper, 1985; Faden, 1993; Siesjo et al., 1995). Alterations in excitatory amino acids (EAA), increased oxidative stress (ROS), and the disruption of Ca^{2+} homeostasis are major factors contributing to the ensuing neuropathology (Braughler et al., 1985; Faden et al., 1989; Choi et al., 1990; Braughler and Hall, 1992). Compelling experimental data also demonstrates that mitochondria play a fundamental role in the death cascade, and mitochondria have been directly linked to EAA-mediated neurotoxicity (Nicholls and Budd, 1998a; Stout et al., 1998; Jiang et al., 2001; Brustovetsky et al., 2002; Sullivan et al., 2003). The present studies are based on the hypothesis that injury-induced glutamate release increases mitochondrial Ca^{2+} cycling/overload ultimately leading to mitochondrial dysfunction and that transient mitochondrial uncoupling can confer neuroprotection following traumatic brain (TBI) and spinal cord (SCI) injuries.

Extrinsic mitochondrial uncouplers are compounds that facilitate the movement of protons from the mitochondrial inner-membrane space into the mitochondrial matrix. Intrinsic uncoupling can be mediated via the activation of endogenous mitochondrial uncoupling proteins (UCP) which utilize free fatty acids to translocate protons. This short circuit "uncouples" the pumping of protons out of the matrix via the electron transport system (ETS) from the flow of protons through the ATP synthase and results in a coincidental reduction in the mitochondrial membrane potential ($\Delta\Psi$). While it is obvious that long-term, complete uncoupling of mitochondria would be detrimental, a transient or "mild uncoupling", could confer neuroprotection. Mild uncoupling during the acute phases of injured-induced excitotoxicity would be expected to reduce mitochondrial Ca^{2+} uptake (cycling) and ROS production, since both are $\Delta\Psi$ -dependent.

Following TBI and SCI, there is a significant loss of mitochondrial homeostasis, resulting in increased mitochondrial ROS production and disruption of synaptic homeostasis (Azbill et al., 1997; Xiong et al., 1997; Sullivan et al., 1998; Sullivan et al., 1999b; Sullivan et al., 1999a). This implicates an underlying pivotal role for mitochondria in the sequelae of injury-related neuropathology. Our laboratories and others have solidified this theory by demonstrating that therapeutic intervention with cyclosporin A following experimental TBI significantly reduces mitochondrial dysfunction (Sullivan et al., 1999a) and cortical damage (Scheff and Sullivan, 1999; Sullivan et al., 2000c; Sullivan et al., 2000a), as well as cytoskeletal changes and axonal dysfunction (Okonkwo et al., 1999; Okonkwo and Povlishock, 1999). At least part of the mechanism by which CsA affords neuroprotection is via the maintenance of mitochondrial homeostasis by inhibiting the opening of the mitochondrial permeability transition pore (Buki et al., 1999; Okonkwo et al., 1999; Okonkwo and Povlishock, 1999; Scheff and Sullivan, 1999; Sullivan et al., 1999a). Furthermore, maintaining mitochondrial bioenergetics by dietary supplementation with creatine has also proved effective in ameliorating neuronal cell death and reduces mitochondrial ROS production and maintaining ATP levels following TBI (Sullivan et al., 2000b).

Although the complex mechanisms of secondary neuronal injury are poorly understood, it is clear that EAA neurotoxicity plays an important role (Rothman and Olney, 1995). This results in excessive entry of Ca^{2+} , leading to a loss of cellular homeostasis and subsequent neuronal Ca^{2+}



overload. Ca^{2+} is the most common signal transduction element in cells, but unlike other second-messenger molecules, it is required for life. Paradoxically, prolonged high levels of $[\text{Ca}^{2+}]_i$ leads to cell death (Choi, 1992). Since Ca^{2+} cannot be metabolized like other second-messenger molecules, it must be tightly regulated by cells. Numerous intracellular proteins and some organelles have adapted to bind or sequester Ca^{2+} to ensure that homeostasis is maintained. *Mitochondria are one such organelle* (Ichase and Mazat, 1998; Rizzuto et al., 1999; Rizzuto et al., 2000). During excitotoxic insult, Ca^{2+} uptake into mitochondria has been shown to increase ROS production, inhibit ATP synthesis and induce mitochondrial permeability transitions (Dugan et al., 1995; Reynolds and Hastings, 1995; White and Reynolds, 1996; Sengpiel et al., 1998; Brustovetsky et al., 2002). It is also important to note that inhibition of mitochondrial Ca^{2+} uptake by reducing $\Delta\Psi$ (chemical uncoupling) following excitotoxic insults is neuroprotective, emphasizing the pivotal role of mitochondrial Ca^{2+} uptake in EAA neuronal cell death (Nicholls and Budd, 1998a, b; Stout et al., 1998).

Free radical production is a byproduct of ATP generation in mitochondria via the electron transport chain. Electrons escape from the chain and reduce O_2 to $\text{O}_2^{\cdot-}$. Normally cells convert $\text{O}_2^{\cdot-}$ to H_2O_2 utilizing both manganese superoxide dismutase, which is localized to the mitochondria, and copper-zinc superoxide dismutase found in the cytosol. H_2O_2 is rapidly converted to H_2O via catalase and glutathione peroxidase, but has the potential to be converted to the highly reactive hydroxyl radical (OH^{\cdot}) via the Fenton reaction, underlying ROS neurotoxicity. OH^{\cdot} rapidly attacks unsaturated fatty acids in membranes causing lipid peroxidation and the production of 4-hydroxynonenal (HNE) that conjugates to membrane proteins, impairing their function (Azbill et al., 1997; Keller et al., 1997; Keller et al., 1997; Mark et al., 1997; Sullivan et al., 1998). In particular, ROS induction of lipid peroxidation and protein oxidation products may be particularly important in neurodegeneration (for review see (Mattson, 1998)) and TBI (Braugher et al., 1985; Braugher and Hall, 1989, 1992; Sullivan et al., 1998).

Mitochondrial ROS production is intimately linked to $\Delta\Psi$ such that hyperpolarization (high $\Delta\Psi$) increases and promotes ROS production (Skulachev, 1996, 1998; Votyakova and Reynolds, 2001; Liu et al., 2002; Starkov et al., 2002; Starkov and Fiskum, 2003). Since the magnitude of ROS production is largely dependent on—and correlates with $\Delta\Psi$ even a modest reduction via increased proton conductance (decreases $\Delta\Psi$, the electrochemical proton gradient) across the mitochondrial inner membrane (uncoupling) reduces ROS formation (Skulachev, 1996; Kim-Han et al., 2001; Votyakova and Reynolds, 2001; Sullivan et al., 2003; Sullivan et al., 2004b).

Endogenous mitochondrial uncoupling is mediated by members of the UCP family, which function to dissociate ATP production from oxygen consumption in mitochondria of muscle and fat tissues (Nicholls and Ward, 2000), leading to heat generation. UCPs are activated by FFAs, superoxide and inhibited by purine nucleotides (Echtay et al., 2002) (also see (Harper et al., 2001; Argiles et al., 2002; Zackova and Jezek, 2002) for review). Five mitochondrial UCPs exist in the human genome and among characterized uncoupling proteins, UCP2, UCP4 and UCP5/BMCP1 have recently been shown to be significantly expressed in the CNS (Horvath et al., 1999; Arsenijevic et al., 2000; Diano et al., 2000; Kim-Han et al., 2001). However, unlike UCP1 which is present only in brown adipose tissue and used to generate heat in cold environments (i.e. thermogenesis), their physiological role(s) are unclear.

Several hypotheses have been put forth concerning possible physiological roles of the UCPs including energy partitioning, energy balance and control of metabolism which may be pivotal in obesity and diabetes (for review see (Argiles et al., 2002; Jezek, 2002)). Skulachev was the first to hypothesize that mild uncoupling could be beneficial since it causes a decrease in ROS production ((Skulachev, 1996) and preceding section). Several studies have now demonstrated roles for UCPs in modulating ROS production. UCP2 (Arsenijevic et al., 2000) or UCP3 (Vidal-Puig et al., 2000) knockout mice exhibit increased ROS in macrophages and muscle, respectively. Leptin-deficient mice have decreased levels of UCP2 and increased ROS production in macrophages (Lee et al.,



1999). Overexpression of UCP2 (Li et al., 2001) or UCP5/BMCP1 (Kim-Han et al., 2001) has also been shown to decrease cell death following H_2O_2 exposure and ROS production respectively. UCP2 overexpression has also been demonstrated to reduce ROS production and increase tissue sparing *in vivo* following ischemia or TBI (Mattiasson et al., 2003).

Based on these initial reports, it is reasonable that increasing UCP activity by modulating dietary fat could directly modulate and reduce mitochondrial ROS production and subsequent oxidative damage. We have indeed shown that the converse (i.e. reducing dietary fat in immature animals) does rapidly reduce neuronal UCP expression/activity and increases mitochondrial ROS production. These changes in mitochondrial UCP activity and ROS production decrease the resistance of these immature animals to excitotoxic insult resulting in increased neuronal cell death following seizure activity, implicating a neuroprotective role for UCP2 and mitochondrial uncoupling in neuronal injury (Sullivan et al., 2003). These data also suggest that increasing dietary fat content would increase UCP activity and reduce ROS production, both of which we have recently demonstrated to occur *in vivo* (Sullivan et al., 2004a).

Several studies have demonstrated that mitochondrial uncoupling *in vitro* reduces neuronal mitochondrial Ca^{2+} loading and can inhibit excitotoxic cell death (Nicholls and Budd, 1998a, b; Stout et al., 1998; Billups and Forsythe, 2002; Pivovarova et al., 2002). To date only one study has assessed the potential for using mitochondrial uncouplers (2,4-DNP) as neuroprotective agents in an *in vivo* model of excitotoxicity (Maragos et al., 2003). Since it is well-established that excitotoxicity is a major player in TBI- and SCI-induced neuronal cell death and results in significant mitochondrial dysfunction, we designed several experiments to test the hypothesis that the mitochondrial uncouplers 2,4-DNP and FCCP would be neuroprotective following TBI and SCI.

The results demonstrate that rats administered a mitochondrial uncoupler have less tissue loss, improved behavioral outcomes and demonstrate a reduction in mitochondrial oxidative damage, Ca^{2+} loading and dysfunction following SCI or TBI. The results also demonstrate that mitochondrial uncouplers significantly reduces mitochondrial dysfunction associated with injury whereas a 2,4-DNP analogue 2,4,6-trinitrophenol (TNP), which lacks the ability to uncouple intact mitochondria, did not provide any neuroprotection. Importantly, post-injury fasting of animals (24 hrs) following TBI yields similar results, perhaps by utilizing endogenous mitochondrial uncoupling proteins (UCP). Together these results implicate important mitochondrial events that could be potential novel interventions and novel targets for the treatment of TBI and SCI as well as other acute neuronal injuries.

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Regulation of synaptic transmission by mitochondrial ion channels

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Regulation of events at the presynaptic terminal of a synapse is important for determining whether a neuronal pathway will become strengthened during such processes as learning and the making of new memories. Conversely, biochemical events at the synapse can cause the synapse to fail during neurodegeneration, such as in Alzheimer's disease, or in acute injury, such as during brain ischemia. Studies of the modulation of synaptic transmission comprise an important area of focus in the field of Neurobiology.

The squid giant presynaptic terminal is a well-established model system for studying neurotransmission. It has an extremely large (1 mm) presynaptic terminal that enables investigators to study synaptic properties with relative ease. Electron micrographs reveal collections of synaptic vesicles adjacent to the area of contact between pre and postsynaptic cells (Martin, 1975, Jonas et al., 1999). Deeper inside the terminal are arrays of neurofilaments and numerous mitochondria that are thought to provide the energy for neurotransmission and to manage the calcium that enters at the active zone. Most other types of organelles are absent. In mammalian synapses, presynaptic mitochondria have specific morphological features that differentiate them from other types of mitochondria (Tolbert and Morest, 1982). For example, some presynaptic mitochondria are tethered to active zones and physically linked to chains of vesicles (Rowland et al., 2000).

Previous studies have begun to shed light on the role of mitochondria in the synapse, and have hinted that, rather than simply making ATP constitutively to provide the energy for synaptic activity, they regulate the supply of ATP. Mitochondria are also known to buffer cytoplasmic calcium ions. Sustained elevations in presynaptic calcium following rapid, repetitive neuronal firing are not only correlated with enhancement of synaptic transmission (Swandulla et al., 1991; Wang and Kaczmarek, 1998), but also require intact mitochondria in secretory cells (Babcock and Hille, 1998) and in neurons (Friel and Tsien, 1994; Nguyen et al., 1997; Tang and Zucker, 1997; Billups and Forsythe, 2002). Nevertheless, the specific molecular mechanisms that define the role of mitochondria in calcium and metabolite management during high frequency presynaptic activity are not yet known.

Intracellular ion channel recording technique

The activity of mitochondrial ion channels is required for calcium buffering and release of energy metabolites. To characterize channels on mitochondrial membranes that might be important during synaptic transmission and during apoptosis, we developed a technique to record from intracellular ion channels in intact cells (Jonas et al. 1997b, Jonas et al. 1999). The intracellular organelle recording technique is a variant of the patch clamp technique but the patch electrode is contained within an outer, large bore microelectrode. The concentric electrodes can be manipulated past the plasma membrane, after which the outer electrode is withdrawn, exposing the inner tip. Negative pressure causes the inner tip to form a high resistance seal on intracellular membranes, after which single channel data is gathered either on the organelle, or after excision of the patch into the cytoplasm or bath. Lipophilic fluorescent dyes (Pagano et al., 1989) have been included in the patch pipette, and give information about the intracellular location of the pipette tip. In the squid presynaptic terminal, as in many other presynaptic terminals, mitochondria are the only internal organelles that are compatible with seal formation by the patch pipettes, which have tip diameters of approximately ~180-200 nm by scanning electron microscopy.



Effects of synaptic stimulation on mitochondrial ion channel activity

Mitochondrial recordings inside the resting squid presynaptic terminal reveal small conductance activity. Very infrequently, much larger conductances occur spontaneously. Electrical stimulation of the squid presynaptic terminal to evoke synaptic transmission, however, causes a marked change in activity and conductance of mitochondrial patches. During a brief, high frequency, train of stimuli, mitochondrial ion channel activity increases inside the terminals, resulting in an approximately 60-fold enhancement of membrane conductance lasting up to 60 seconds (Jonas et al., 1999). Activity then gradually decreases in frequency and amplitude over the 5 to 30 s following the period of enhanced activity.

The delayed onset and the persistence of the mitochondrial channel activity after stimulation implies that this increased activity on the mitochondrial outer membrane is not simultaneous with the opening of plasma membrane channels, and suggests that this increase may depend on an intracellular second messenger. During synaptic stimulation, there is a build-up of calcium in the presynaptic terminal. This is thought to be responsible for a form of short term synaptic plasticity termed posttetanic potentiation, and this persistent calcium elevation has been found previously to depend on mitochondria (Tang and Zucker, 1997; Friel and Tsien, 1994). In recordings from mitochondrial membranes inside the terminal in a calcium-deficient bathing medium, there is no response to stimulation of the presynaptic nerve, demonstrating that the evoked intracellular membrane channel activity is dependent on calcium influx. Intracellular membrane channel activity is also dependent on an intact mitochondrial membrane potential. Uncoupling mitochondria with FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) completely eliminates the increase in conductance during stimulation. FCCP also eliminates posttetanic potentiation. The timing of the changes in mitochondrial conductance and their dependence on calcium suggest that opening of a mitochondrial channel is important for short term plasticity of the synapse.

The identities of the mitochondrial channels responsible for changes during synaptic transmission are not yet known. The channels of interest that may undergo regulation during synaptic events include those of both the inner and outer membranes. The calcium-selective uniporter (Kirichok et al., 2004), and the calcium sensitive permeability transition pore (Bernardi, 1996) are candidates for inner membrane channels. Once calcium and metabolites are released from the matrix into the intermembrane space, they are released across the outer membrane to reach the cytosol. The voltage dependent anion channel (VDAC), which is a ubiquitous protein in mitochondrial outer membrane (Colombini et al., 1996) may perform this function. More recently, channels formed by interactions of BCL-2 family proteins with mitochondrial membranes (Kroemer, 1997; Reed, 1997) have been found to release or inhibit the release of mitochondrial components such as cytochrome c into the cytosol. Many BCL-2 family proteins, such as BCL-xL, are endogenously present in mitochondrial membranes (Kaufmann et al., 2003). BCL-xL is a potent inhibitor of programmed cell death and is abundantly expressed in neurons of the adult brain (Boise et al., 1993; Krajewski et al., 1994; Gonzalez-Garcia et al., 1995; Frankowski et al., 1995; Blomer et al., 1998) where its role in developmental apoptosis is obviously constrained. It has been suggested that the role of BCL-xL in adult neurons is to protect cells from death by regulating export of ATP from mitochondria and/or by blocking the activation of pro-apoptotic proteins (Basanez et al., 2002; Vander Heiden et al., 2000; Zong et al., 2001), but other roles for this important molecule have yet to be elucidated.

Although BCL-2 family proteins are able to conduct ions when reconstituted into artificial lipid bilayers (Schendel et al., 1998; Minn et al., 1997; Antonsson, et al., 1997; Schlesinger et al., 1997), the precise biochemical mechanisms by which they regulate mitochondrial permeability and apoptosis in cells or whether they form channels *in vivo* was not previously known.



BCL-xL in the presynaptic terminal of the squid giant synapse enhances synaptic transmission and induces channel activity in mitochondria.

BCL-xL is present on mitochondria in the stellate ganglion of the adult squid (Jonas et al., 2003), where it could play a role in protection against acute insults to the nervous system. To determine the action of BCL-xL on mitochondrial membrane conductances, we recorded from mitochondria inside the synapse with recombinant BCL-xL in the patch pipette solution (Jonas et al., 2003). Activity with multiple conductances was readily detected in these mitochondrial patches (Fig.1). Activity typically switched between different conductance levels every few seconds, but a single conductance level could also occasionally be maintained for several minutes. Recordings made with BCL-xL in the patch pipette demonstrated larger conductances than those observed in control recordings.

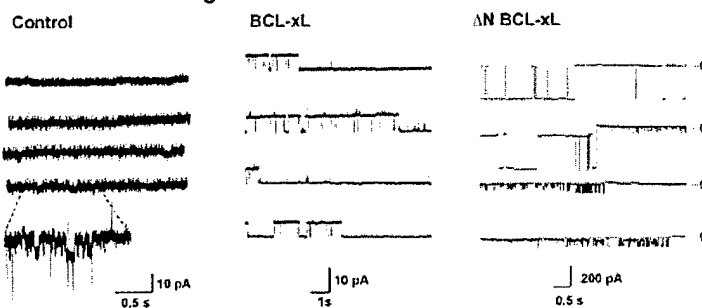


Figure 1. Different forms of multiconductance channel activity are produced by the anti-apoptotic protein BCL-xL and its pro-apoptotic cleavage fragment Δ N BCL-xL applied to mitochondria within the presynaptic terminal of the squid. Left panel shows small conductance channel activity in control mitochondria.

Synaptic activity on mitochondrial membranes could be a consequence of, or an integral link in the chain of events that lead to posttetanic potentiation. Because BCL-xL produces a change in mitochondrial membrane conductance, it is a possible candidate for a mitochondrial membrane channel that could alter synaptic transmission. Indeed, injection of BCL-xL into presynaptic terminals enhances the rate of rise of postsynaptic responses in both healthy synapses and in those in which transmission has run down to the point that the postsynaptic potential no longer triggers postsynaptic action potentials.

ATP enhances synaptic transmission.

Work with non-neuronal cells has suggested that BCL-xL regulates the flux of metabolites across the outer mitochondrial membrane to facilitate transport of ATP into the cytosol following a death stimulus (Vander Heiden et al., 2000, 2001). Consistent with these findings, in squid synapses terminals, direct microinjection of ATP into the presynaptic cell effectively enhances the post-synaptic responses, and injected ATP occludes the effects of BCL-xL. The findings raise the possibility that BCL-xL may enhance synaptic activity by triggering release of ATP from mitochondria, and support the idea that the ion channel function of anti-apoptotic proteins may include regulation of the release of ATP. The moderate size of the conductance (200-500 pS) produced by the anti-apoptotic protein in mitochondrial membranes suggests that it would be unable to release large components of mitochondria such as cytochrome c during apoptosis.

Induction of large conductances during insults to the synapse

In apoptosis or in insults to the nervous system, a different set of changes occur in the outer membrane. These are associated with the release of cytochrome c and other factors from the intermembrane space (Gross et al., 1999). Under these conditions BCL-2 family proteins, either by interaction with VDAC or by independent mechanisms, may also contribute to channel activity in the outer membrane without any activation of the inner membrane (Polster et al., 2001). In order to study how mitochondria participate in pathological states of the synapse that might be similar to apoptotic conditions, and how the outer membrane channels VDAC and BCL-xL participate in these events, we studied the reverse of synaptic potentiation, synaptic rundown during hypoxia.



Hypoxia induces multiconductance channel activity in synaptic mitochondria.

We used the giant synapse of the squid stellate ganglion as a model system to study the effects of hypoxia on mitochondrial ion channel activity. The presynaptic terminal of this synapse is very sensitive to hypoxia, which attenuates synaptic transmission over 10- 30 minutes (Fig.2).

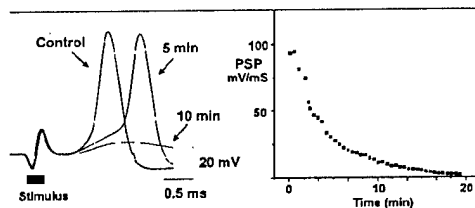


Figure 2. Hypoxia causes synaptic rundown. Postsynaptic responses are shown at different times after the onset of oxygen deprivation. The second panel shows the change in slope of postsynaptic potentials over time after the onset of oxygen deprivation.

Hypoxia can be induced in squid giant stellate ganglia by eliminating perfusion of oxygenated sea water. Patch clamp recordings of channel activity on mitochondria during hypoxia show that, in contrast to controls, a new, large multiconductance channel appears on average within about 13 minutes after the start of hypoxia. Conductances ranging from 300 pS to 2.0 nS can be detected in these hypoxic neurons using invertebrate intracellular solution.

The channel activity produced by hypoxic conditions is much larger than that observed with application of full length BCL-xL to the patch, but closely resembles that produced by applying a pro-apoptotic version of BCL-xL to the mitochondrial membranes. Whether BCL-xL, which we have shown to be correlated with potentiation of the synapse, could also be responsible for synaptic rundown during hypoxia, is an interesting question. BCL-xL protein can be cleaved between the BH4 and BH3 domains by zVAD-sensitive proteases caspase-3 (Asp61, Asp76) and calpain (Ala60) to produce a pro-apoptotic C-terminal fragment, N BCL-xL lacking amino acids 2-76 (Clem et al., 1998; Fujita et al., 1998; Nakagawa and Yuan, 2000). In the squid presynaptic terminal, hypoxia produces proteolysis of BCL-xL, an effect that can be blocked by the protease inhibitor zVAD.

In mammalian cells, overexpression of Δ N BCL-xL potently induces loss of mitochondrial membrane potential, cytochrome c release from mitochondria and apoptosis (Kirsch et al., 1999; Basanez et al., 2001). Injection of Δ N BCL-xL into the squid presynaptic terminal has the opposite effect from that of full length BCL-xL, it attenuates synaptic transmission (Jonas et al., 2003). In addition, with the same time course as that for the onset of rundown of transmission, application of Δ N Bcl-xL to mitochondrial membranes in intact terminals produces large multiconductance channel activity, similar to that seen during hypoxia. This activity is dependent on an intact BH3 domain and on interaction with mitochondrial membranes. Peak conductances in different recordings range between 300 pS and 3.8 nS, similar to channel activity observed during hypoxia. The large conductance of this channel could be responsible for the release of cytochrome c and other pro-apoptotic factors into the cytosol during hypoxic or degenerative synaptic rundown. Formation of Δ N BCL-xL mitochondrial channels requires VDAC.



Because the gigaohm seals were formed directly on intracellular organelles within the presynaptic terminal, it is likely that the membrane contacted by the patch pipette is the outer mitochondrial membrane. The conductance of this membrane is known to be reduced by millimolar concentrations of NADH (Lee et al., 1994; Wunder and Colombini, 1991). In lipid bilayers, NADH has also been shown to reduce the conductance of VDAC, a relatively non-selective channel that is believed to be the major conductance pathway across the outer membrane. In squid, NADH specifically reduces the probability of large conductance activity induced by \square N BCL-xL or hypoxia in mitochondrial membranes. It fails, however, to inhibit the actions of \square N BCL-xL on the permeability of artificial lipid membranes. Taken together, the data suggest that NADH alters the conductance of hypoxia- or \square N BCL-xL-induced channels by acting on a mitochondrial component other than the BCL-xL protein alone.

To further investigate the hypothesis that \square N BCL-xL or hypoxia require VDAC to form channels, recordings were made on mitochondrial membranes prepared from wild type yeast and from yeast that lack the *por1* gene, which encodes the VDAC-1 channel (YVDAC1) (Lohret and Kinnally, 1995; Lee et al., 1998).

Recording from the outer membrane of isolated wild type yeast mitochondria revealed a voltage-dependent behavior with properties similar to that previously described for VDAC in artificial membranes (Colombini et al., 1996). In a separate set of recordings on these wild type mitochondria, the inclusion of \square N BCL-xL protein in the pipette solution resulted in a very different pattern of activity. The typical VDAC-like activity could no longer be detected, and significantly larger conductance activity was detected at both positive and negative potentials. These large conductances were similar to those observed after the addition of \square N BCL-xL in squid presynaptic terminal, and were also markedly attenuated by the addition of NADH.

Large conductance activity could be recorded under control conditions in mitochondrial membranes from mutant \square POR1 yeast lacking the VDAC channel, but, in contrast to the wild-type mitochondria, this activity was completely unaffected by \square N BCL-xL and was not inhibited by NADH, suggesting that VDAC is required for formation of channels by the \square N BCL-xL protein.

In summary, by using techniques that allow mitochondrial membrane recordings within living cells, we can begin to describe mitochondrial ion channel activities that play a role in synaptic transmission.

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Mitochondrial dysfunction contributes to cell death following traumatic brain injury in adult and immature animals

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Approximately 1.5 million people sustain traumatic brain injury (TBI) in the United States each year (CDC). Of these, over 50,000 patients die annually, accounting for greater than 1/3 of all injury-related deaths (Sosin 1995). Among survivors, almost 1/4 million people sustain a degree of injury that warrants hospitalization. In 1995, it was estimated that the total direct and indirect financial costs of TBI-related injuries were greater than \$50 billion (Thurman 2001). The long-term social and emotional burden may be even greater, with an estimated 5.3 million men, women and children living with a TBI-related disability (CDC, 1999). Despite the significant public health impact of TBI, limited neuroprotective interventions exist for those suffering severe TBI.

Mitochondria and TBI – Preclinical studies

Growing evidence suggests an important role for mitochondria as subcellular targets for neuroprotection after TBI. Factors that both inhibit and promote neuronal apoptosis appear to work by influencing mitochondrial cytochrome c release, and pathways that promote necrotic cell death, such as excitotoxicity and oxidative stress, have profound influences on mitochondrial function (Figure 1). Importantly, both preclinical and clinical studies have documented apoptotic and necrotic neural cell death after TBI.

Although the importance of mitochondria following brain injury has been suggested and studied for many years (reviewed in Fiskum et al., 1999), the majority of studies describing mitochondrial roles specifically in TBI have been conducted in the last decade. Early studies evaluated changes in mitochondrial respiration in the first few hours after injury and found slight reductions in the active, phosphorylating rate of mitochondrial respiration 4 hours after fluid percussion TBI (Vink et al., 1990). Subsequent studies using the controlled cortical impact model of TBI defined more dramatic alterations in mitochondrial respiration that began within 1 hour of injury and persisted for at least 14 days (Xiong et al., 1997a). In addition, mitochondria isolated from the hemisphere ipsilateral to injury demonstrated reduced ability to sequester Ca^{2+} (Xiong et al., 1997a). These alterations in mitochondrial respiration and Ca^{2+} transport were reversible by post-injury treatment with the calcium channel blocker, SNX-111 (Verweij et al., 1997) and the antioxidant, U-101033E (Xiong et al., 1997b), both alone and in combination (Xiong et al., 1998). These studies emphasize the potential for neuroprotection after TBI through pharmacologic intervention that directly targets mitochondrial dysfunction.

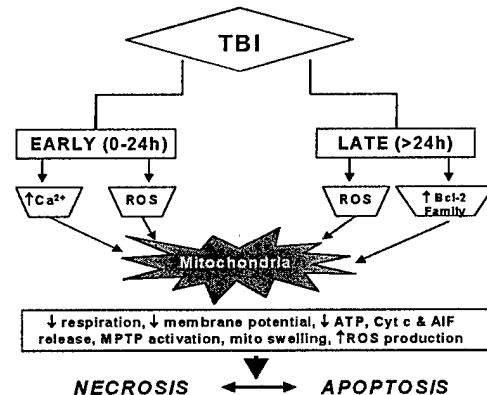


Figure 1. Mechanisms of mitochondrial injury after TBI. TBI results in both early and delayed neural injury, both of which can have a profound influence on mitochondrial function and ultimately lead to necrotic and apoptotic cell death.



In addition to perturbations in mitochondrial respiration and Ca^{2+} homeostasis, TBI has recently been shown to have effects on mitochondrial membrane potential. Isolated mitochondria and synaptosomes from injured cortex show reduced membrane potential and evidence of mitochondrial inner membrane permeability changes (Sullivan et al., 1999). Membrane potential was restored by the administration of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore (MPTP). Similar types of mitochondrial abnormalities involving alterations in membrane potential with resultant mitochondrial swelling have also been seen in models of TBI examining axonal injury (Pettus and Povlishock, 1996). This series of investigations has described a key role for loss of mitochondrial integrity in the secondary axotomy that occurs following TBI (Okondkwo et al., 1999; Buki et al., 1999; Okonkwo and Povlishock, 1999).

Another important pathway with great significance in TBI is mitochondrial cytochrome c release and resultant neuronal apoptosis (reviewed in Raghupathi et al., 2000). Experimental studies of TBI have demonstrated mitochondrial cytochrome c release in many models of TBI, including cold injury-induced brain trauma (Morita-Fujimura et al., 1999), traumatic axonal injury (Buki et al., 2000), and controlled cortical impact (Lewen et al., 2001; Sullivan et al., 2002). Downstream events, such as caspase activation, have also been well documented in animal models of TBI (Yakovlev et al., 1997; Clark et al., 2000; Keane et al., 2001; Knoblach et al., 2002; Sullivan et al., 2002).

Mitochondria and TBI – Clinical studies

Although the number of studies directly evaluating mitochondrial function after TBI in humans is limited, they have generally supported findings seen in animal models of TBI. Brain mitochondria isolated from human victims of TBI have shown impaired rates of respiration and ATP synthesis (Verweij et al. 1997; Verweij et al., 2000). One interesting study of human autopsy tissue compared brain mitochondrial DNA deletions in short-term survivors of cardiac arrest, long-term survivors of TBI and age-matched controls, evaluating the role of mitochondrial gene expression in brain injury (McDonald et al., 1999). They discovered a significantly lower incidence of mitochondrial DNA deletions in long-term survivors of TBI and hypothesized that chronic free radical-induced mitochondrial DNA damage may ultimately influence the survival of TBI victims.

A few clinical studies have demonstrated evidence for apoptotic cell death involving mitochondrial pathways after TBI in adults and children. For example, caspase activation was documented in human TBI tissue (Clark et al., 1999), and bcl-2 protein was increased in brain tissue from adult patients and in cerebrospinal fluid (CSF) from pediatric patients after TBI (Clark et al., 1999; Clark et al., 2000). Very recent clinical studies in pediatric TBI have discovered elevations in two mitochondrial proteins, heat shock protein 60 (Hsp60) and cytochrome c, in the CSF of head-injured children compared to non-injured pediatric controls (Stange et al., 2003; Lai et al., 2003). In these studies Hsp60 correlated with injury severity and cytochrome c levels correlated with child abuse victims and female gender. The presence of these integral mitochondrial proteins in the CSF suggests mitochondrial damage in these patients, and the correlation with demographic features may prove to be helpful in defining subpopulations likely to respond to specific neuroprotective interventions. Ongoing investigation into the degree and features of mitochondrial dysfunction after TBI in humans is warranted, and could lead to the development of novel, subcellular, neuroprotective strategies aimed at early and sustained mitochondrial impairment.

Mitochondria and the Developing Brain

Developmental differences in brain mitochondria of normal rats have been well documented. In general, through the first 3-4 weeks of life in the rat, there is a 3-fold increase in mitochondrial protein per cell, with corresponding increases in respiratory enzyme activity and increasing oxygen consumption (Murthy and Rappoport, 1963; Milstein et al., 1968). There are also potential differences in mitochondrial membrane composition (Sitkiewicz et al., 1982) and relative ratios of synaptosomal to non-synaptosomal brain mitochondria (Dienel et al., 1977). A series of studies by



the laboratory of Holtzman and others have detailed developmental differences in brain mitochondrial activity in immature (< 4 weeks old) versus mature (adult) rats. ADP/O ratios with NAD-linked substrates were lower in rats < 2 weeks of age, increased between the 3rd and 4th week, and reached adult levels by the 4th week of life (Holtzman and Moore, 1973 & 1975).

In order to understand the role of mitochondrial dysfunction after injury to the developing brain, one must understand developmental aspects of mitochondrial function in normal (uninjured) brain. Initial studies in our lab have compared brain mitochondria isolated from immature rats to those isolated from adult rats. We evaluated Ca^{2+} uptake capacity of isolated mitochondria in both

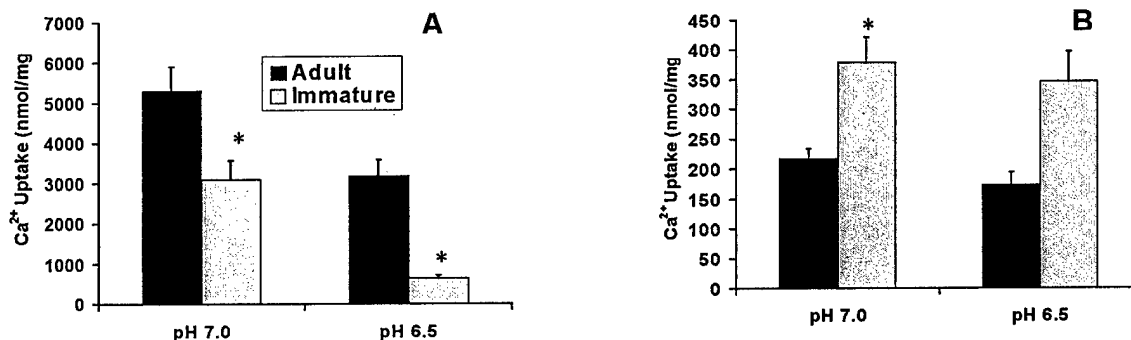


Figure 2. Calcium uptake capacity of mature and immature brain mitochondria. In the presence of 3 mM ATP (Figure 2A), maximal Ca^{2+} uptake capacity is greater in adult (black bars) versus immature (gray bars) rat brain mitochondria at both pH of 7.0 and 6.5. In the absence of ATP (Figure 2B), maximal Ca^{2+} uptake capacity is greater in immature rat brain mitochondria.

physiologic conditions and in those conditions potentially present after TBI, such as acidosis (pH=6.5) and ATP depletion. The Ca^{2+} uptake capacity represents resistance to Ca^{2+} -induced mitochondrial injury. In a physiologic environment (pH=7.0 with ATP), mitochondria isolated from adult rats had a higher Ca^{2+} uptake capacity than mitochondria from immature rats (Figure 2A). Acidosis (pH=6.5) caused a significant reduction in maximal Ca^{2+} uptake in both immature and adult rat brain mitochondria. As brain tissue acidosis contributes to poor outcome following TBI in both animals and humans, these observations suggest a subcellular mechanism of action that could be particularly important in immature animals and children. In contrast to the differences seen with pH modification, immature rats appear to tolerate the absence of ATP much better than adult rats. At both a pH of 7.0 and 6.5, immature rat brain mitochondria had a greater Ca^{2+} -uptake capacity than adult rat brain mitochondria (Figure 2B). These results suggest that brain mitochondria from immature animals are more resistant than those of mature animals to Ca^{2+} -induced injury under extreme conditions (no ATP) that can occur within some brain cells following TBI.

Animal models of hypoxia-ischemia and traumatic brain injury have shown developmental differences in apoptotic neuronal death. The exact mechanisms to explain these differences are unknown, but are likely multifaceted and related to mitochondrial response to injury. To begin to evaluate this, we studied the in vitro response of isolated brain

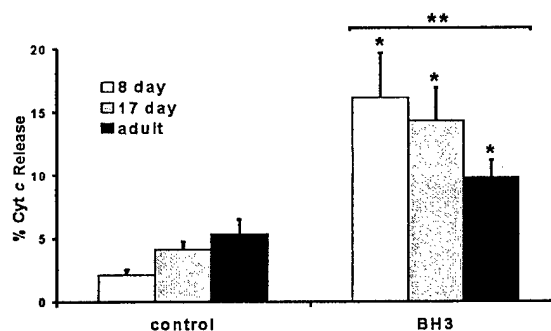
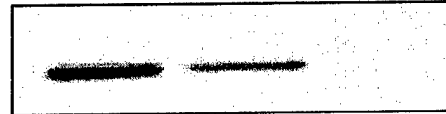


Figure 3. Release of cytochrome c from brain mitochondria isolated from 8-day old, 17-day old, and adult rats in response to BH3 peptide. There is a significant difference between control and BH3 peptide treatment across groups and a significant affect of age on this difference (two-way ANOVA, $p < 0.05$)



mitochondria to pro-apoptotic peptides (BH3 cell death domain-containing peptide). Analysis by ELISA revealed greater cytochrome c release from mitochondria of immature rats exposed to BH3 peptide compared to adult rats, with the youngest rats (8do) showing the greatest release (Figure 3). Previous studies in other laboratories have suggested that the protein Bax may be required for 'BH3 only' proteins to promote cytochrome c release (Desagher et al., 1999; Wei et al., 2001), and brain levels may decline during maturation. Using immunoblot analysis, we found significant amounts of detectable Bax in 8 do isolated rat forebrain mitochondria and moderate

8 do 17do Adult



Bax

Figure 4. Immunoblot for Bax in isolated rat brain mitochondria from 8 day-old, 17 day-old and adult rats.

amounts of detectable Bax in 17 do rats, but none detectable in adult rat brain mitochondria (Figure 4). The presence of endogenous Bax in association with brain mitochondria may represent a potential explanation for the differences observed in sensitivity to BH3-induced cytochrome c release. The potentially very important conclusion we have reached from these results is that immature brain mitochondria are "primed" to release cytochrome c in response to BH3 domain proteins (e.g., tBid) due to the presence of endogenous mitochondrial Bax (Polster et al., 2003). These characteristics of immature brain mitochondria could help explain the apparently greater contribution of apoptosis to brain cell death following TBI in immature animals.

Mitochondrial dysfunction after experimental TBI in immature rats

Given the important role that mitochondria likely play after TBI, and the unique aspects of mitochondrial development in the immature brain, it stands to reason that mitochondrial dysfunction following TBI in the immature brain would have profound effects. Very few studies have addressed this, although several centers have demonstrated unique patterns after TBI in immature rats, and explanations have discussed mechanisms with importance to mitochondrial function, such as alterations in CBF (Grundl et al., 1994; Biagas et al., 1996) and metabolism (Thomas et al., 2000). The most comprehensive investigation to date reveals two unique patterns of cell death after TBI in 7 do rats, involving excitotoxic and apoptotic mechanisms (Pohl et al., 1999). Neurons adjacent to the site of impact showed changes identical to those induced by glutamate, which peaked at 4 h and was not evident by 24 h. A delayed pattern of apoptotic cell death peaked at 24 h, and accounted for a much greater number of dying cells (2.2 million) than excitotoxicity (16,000). Interestingly, NMDA receptor antagonists protected against the primary excitotoxicity, but increased the severity of secondary apoptotic damage. Administration of SPBN, a free radical scavenger mitigated apoptotic damage. This study clearly demonstrates the importance of independent evaluation of pathologic pathways in the developing brain and supports the potential importance of mitochondrial dysfunction in this unique environment.

Mitochondria and Neuroprotection after TBI

With the growing evidence for mitochondrial participation in traumatic neuronal injury, neuroprotective approaches must include strategies aimed to limit and reverse mitochondrial dysfunction. Interventions that have directly targeted mitochondria, such as calcium channel blockade (Verweij et al., 1997; Xiong et al., 1998; Verweij et al., 2000) and antioxidant administration (Xiong et al., 1997b; Xiong et al., 1998; Xiong et al., 1999) have documented reversibility of this mitochondrial dysfunction. Most importantly, animal studies have demonstrated that "mitoprotective" strategies have translated into neuroprotective strategies in models of TBI. Studies by Verweij et al. (2000) and Berman et al. (2000) initially examined time-window profiles and dose-response curves of the calcium channel blocker Ziconotide after TBI using mitochondrial



outcome measures as endpoints. When the optimal mitochondrial dose was administered, rat showed improvements in motor and cognitive testing from 1 to 42 days after TBI. A number of studies involving the mitochondrial PTP inhibitor, cyclosporin A have shown improvement in both mitochondrial function, cerebral metabolism and tissue damage after TBI (Sullivan et al., 1999; Scheff, et al., 1999; Alessandri et al., 2002). These studies suggest an important role for the mitochondrial PTP after TBI, especially with the lack of efficacy of the immunophilin ligand FK506 in one study (Scheff et al., 1999). However, the calcineurin interaction properties cannot be disregarded, as FK506 does protect against traumatic axonal injury (Singleton et al., 2001). Finally, studies have begun to evaluate the role of uncoupling proteins after brain injury through proposed mechanisms of mild mitochondrial depolarization with resultant reduction in ROS generation. Specifically, overexpression of uncoupling protein-2 has been shown to reduce cortical damage and improve neurologic outcome after TBI in mice (Mattiasson et al., 2003).

Conclusion

The importance of mitochondrial dysfunction following TBI in both preclinical and clinical studies is evident. Also evident, from clinical studies, is the extreme heterogeneity of injury following TBI, which can be influenced by age, gender, injury severity, injury mechanism, brain region and number and degree of secondary insults. From the preclinical studies, cellular and even *subcellular* heterogeneity of alterations in metabolism and bioenergetics after TBI has been seen. Development of neuroprotective treatments must take into consideration this variability, and studies must continue to make attempts to understand the molecular mechanisms responsible for neuronal injury in different settings after TBI. This should include rigorous evaluation of important clinical variables, such as patient age, as interventions that are protective in adult models may be ineffective or even detrimental in pediatric TBI (Pohl et al., 1999). Continued study of mitochondrial participation in TBI may ultimately lead to translation into effective neuroprotective interventions targeted at specific patient profiles.

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Mitochondrial impairment in the developing brain after hypoxia-ischemia

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Abstract

The pattern of cell death in the immature brain differs from that seen in the adult CNS. During normal development, more than half of the neurons are removed through apoptosis, and mediators like caspase-3 are highly upregulated. The contribution of apoptotic mechanisms in cell death appears also to be substantial in the developing brain with a marked activation of downstream caspases and signs of DNA fragmentation. Mitochondria are important regulators of cell death through their role in energy metabolism and calcium homeostasis, their ability to release apoptogenic proteins and to produce reactive oxygen species. We find that secondary brain injury is preceded by impairment of mitochondrial respiration, signs of membrane permeability transition, intra-mitochondrial accumulation of calcium, changes in the bcl-2 family proteins, release of pro-apoptotic proteins (cytochrome C, apoptotic inducing factor) and downstream activation of caspase-9 and caspase-3 after hypoxia-ischemia. These data support the involvement of mitochondria-related mechanisms in perinatal brain injury.

Introduction

Perinatal brain injury subsequent to birth asphyxia remains an important clinical problem. Even though we still lack effective neuroprotective strategies, considerable progress has been made in understanding the pathogenesis of neuronal damage in the immature brain (Vannucci et al. 1997; Johnston et al. 2002).

Thus, it is now well accepted that a cerebral hypoxic-ischemic (HI) event of sufficient severity to deplete tissue energy reserves (primary insult) is often followed by a transient but complete restoration of glucose utilization, ATP and phosphocreatine upon reperfusion/ reoxygenation (Blumberg et al. 1997; Gilland et al. 1998a). Thereafter a secondary decrease of high energy phosphates occurs in parallel with a decrease in tissue utilization of glucose, activation of caspase-3 and DNA fragmentation (Blumberg et al. 1997; Gilland et al. 1998a,b; Puka-Sundvall et al. 2000a). Secondary energy failure develops in most brain regions 6-48 h after the insult in immature animal models.

Several studies indicate that mitochondria play an important role in adult ischemia (Fiskum et al. 1999), but the information about the developing brain is limited. In this brief review, I will describe critical mitochondrial events during the early recovery period and present experimental data in support of that mitochondria may have a critical role in the decision of cellular fate after neonatal HI.

Alterations of mitochondrial morphology, localization and metabolism after HI

We recently found that mitochondria (labelled with COX IV) exhibited a "fibrous" pattern of distribution throughout the soma and processes in normal neurons of the cerebral cortex in 7-day-old rats. However, already 2 h after HI, a more punctate or granular appearance of mainly juxta-nuclear COX IV staining was found (Hallin et al. unpublished) in agreement with Northington et al. (2001). In another study, electron microscopy combined with the oxalate-pyroantimonate technique was used to analyze mitochondrial ultrastructure and intramitochondrial calcium accumulation after HI (Puka-Sundvall et al. 2000b). At 30 min and 3 h after HI, a progressive accumulation of calcium



was detected in the endoplasmic reticulum, cytoplasm, nucleus and, most markedly, in the mitochondrial matrix of neurons. Some mitochondria developed a considerable degree of swelling reaching a diameter of several μm at 3 h of reflow, whereas the majority of mitochondria appeared moderately affected. Chromatin condensation was observed in nuclei of many cells with severely swollen mitochondria with calcium deposits. In conclusion, mitochondrial localization seems to change from a widespread to a more perinuclear distribution after HI, accompanied by mitochondrial swelling and accumulation of calcium in the mitochondrial matrix.

During early recovery after HI high energy phosphates in the cerebral cortex are restored as previously mentioned. During this phase, the 2-deoxyglucose (2-DG) utilization was increased, which correlated with increased levels of tissue lactate (Gilland and Hagberg, 1996), and a depression of mitochondrial respiration (Gilland et al. 1998a). We have also found that post-HI administration of an N-Methyl-D-aspartate (NMDA) receptor antagonist normalized 2-DG utilization, lactate levels, improved mitochondrial respiration and attenuated cortical brain injury (Gilland and Hagberg 1996, 1997; Gilland et al. 1998a,b). These data suggest that NMDA-receptor activation in the early recovery phase depresses mitochondrial respiration with a compensatory increase of anaerobic glucose cycling to lactate, which precedes development of cortical brain injury. Interestingly, a similar pattern of increased glucose use occurred in CNS of asphyxiated infants, particularly in brain regions that were subsequently injured (Blennow et al. 1995). Such an increase in glucose utilization occurred in parallel with marked elevations of glutamate in the cerebrospinal fluid (Hagberg et al. 1993), implying that HI brain injury also in post-asphyxiated infants is preceded by a phase of mitochondrial impairment related to activation of excitatory amino acid receptors.

Membrane permeability transition (MPT)

In isolated mitochondria, MPT is associated with a non-specific permeabilization of the inner mitochondrial membrane, resulting in a dramatic swelling of the mitochondria, followed by rupture of the outer membrane (Ravagnan et al. 2002). Experimental studies have found evidence for MPT after ischemia in the adult brain and MPT blockers have been shown to be potent neuroprotectants (Friberg and Wieloch, 2002). In immature rats, the above mentioned ultrastructural changes are compatible with MPT (Puka-Sundvall, 2001). In order to investigate this further, [^{14}C]2-deoxyglucose (DOG) was administered to control animals, and at various time points after HI, and MPT was measured as entrapment of DOG-6-P in mitochondria (Griffiths and Halestrap, 1995). A significant increase in DOG-6-P in mitochondria indicated that MPT occurred in two phases: a primary MPT after 0-1.5 h and a secondary MPT after 6.5-8 h of reperfusion (Puka-Sundvall et al. 2001). We also found a loss of mitochondrial glutathione during early and late recovery (Wallin et al. 2000), offering additional support of a biphasic increase of mitochondrial permeability after HI. However, in contrast to the adult, the MPT blocker cyclosporine A did not affect brain injury or mitochondrial respiration in the neonatal brain.

Intrinsic apoptotic pathway and caspase activation

Studies performed on cultured cells, cell-free systems or purified mitochondria suggest that mitochondria regulate apoptotic cell death through their capacity to release pro-apoptotic proteins (Ravagnan et al. 2002). Cytochrome c, and other apoptogenic proteins like apoptosis inducing factor (AIF), endonuclease G, SMAC/Diablo and HtrA2/Omi, can be released from the mitochondrial intermembrane space (Ravagnan et al. 2002). Data suggest that Bax, Bad, Bid and other members of the Bcl-2 family are involved in the regulation of mitochondrial release of pro-apoptotic proteins. Cytochrome c interacts with APAF-1, ADP and pro-caspase-9 to form the heptameric apoptosome, leading to activation of caspase-9, which in turn cleaves and activates pro-caspase-3. AIF, on the other hand, promotes apoptosis in a caspase-independent manner (Susin et al. 1999). Many of the key elements of apoptosis have been demonstrated to be strongly upregulated in the immature brain, such as caspase-3 (Blomgren et al. 2001), APAF-1 (Ota et al. 2002), Bcl-2 (Merry



et al. 1994) and Bax (Vekrellis et al. 1997). Caspase-3 is markedly activated after HI in the immature brain (Cheng et al. 1998; Zhu et al. 2000; Wang et al. 2001) and cells with the cleaved active form of caspase-3 co-localize with markers of DNA fragmentation in injured brain regions (Zhu et al. 2000). Caspase-3 inhibitors (Cheng et al. 1998) as well as transgenic overexpression of X-linked Inhibitor of Apoptosis (XIAP) (Wang et al. 2004) attenuate caspase-3 activation and provide a considerable degree of neuroprotection in the neonatal setting.

It is not known if the extrinsic or intrinsic (mitochondrial) pathway is responsible for the downstream activation of caspase-3. However, assembly of the apoptosome is easily induced in homogenates from the immature (but not adult) brain (Gill et al. 2002), cytochrome C is released to the cytosol in response to HI (Northington et al. 2001; Zhu et al. 2003) and caspase-9 is activated (Northington et al. Unpublished; Hallin et al. 2004, unpublished). In addition, other pro-apoptotic proteins like AIF (Zhu et al. 2003), SMAC/Daiblo (Wang et al. 2004) and HtrA2/Omi (Wang et al. 2004) translocate from the mitochondria to a nuclear localization, suggesting that pro-apoptotic proteins are indeed released during the early recovery phase after HI. We find that cells with immunohistochemical translocation of Cytochrome C and AIF often exhibit signs of DNA fragmentation (detected with a hair-pin probe) and nuclear condensation, and these cells are preferentially localized in regions with early loss of the neuronal marker MAP-2 (Zhu et al. 2003). Smac and HtrA2 translocation also occurred predominantly in injured areas and immunostaining often occurred in cells with nuclear condensation or pyknosis (Wang et al. 2004). These data show an association between mitochondrial release of pro-apoptotic proteins and brain injury, but their direct role in the process leading to cell death remains to be clarified.

Bcl-2 family of proteins and neonatal HI

There is also evidence for involvement of the Bcl-2 family of proteins. Transgenic mice overexpressing human Bcl-xL postnatally were dramatically resistant to neonatal HI and axotomy-induced apoptosis (Parsadanian et al. 1998). In addition, HI induced an increase in Bax in mitochondrial-enriched cell fractions, which occurred in parallel with an increase of Cytochrome C in the cytosol preceding activation of caspase-3 in the neonatal thalamus (Northington et al. 2001). Furthermore, HI brain injury seems to be attenuated in Bax gene-deficient mice compared to wild-type controls (Gibson et al. 2001). We recently found that Bax translocation to mitochondria after HI, was accompanied by an increased nuclear staining of Bcl-2 (Hallin et al. Unpublished). Using a site-specific antibody for phosphorylation of Bcl-2 at serine-24 (PS24-Bcl-2), it was found that the number of cells positive for PS24-Bcl-2 increased during 3-24 h of reperfusion in all investigated brain areas after neonatal HI. Phosphorylation of Bcl-2 coincided with Cytochrome C translocation and colocalized with, but preceded, caspase-3 activation. In summary, Bcl-2 is phosphorylated (inactivated ?) and translocated to the nucleus, concomitant with increased mitochondrial Bax immunoreactivity, Cytochrome C release and activation of caspase-3. Furthermore, ceramide "preconditioning-like" protection in the neonatal setting was accompanied by upregulation of both Bcl-2 and Bcl-xL (Chen et al. 2001), offering additional support for involvement of Bcl-2 family proteins and mitochondria in the determination of susceptibility of the immature brain.

PARP-1 and AIF

PARP-1 is a DNA repair enzyme that has been demonstrated to be critically involved in ischemic brain injury in the adult (Yu et al. 2003). Mice with PARP-1 gene disruption are resistant to ischemia (Eliasson et al. 1997) and PARP inhibitors provide protection (Ducrocq et al. 2000; Yu et al. 2003). We recently found that PARP-1 gene deficiency confers protection also in neonatal mice (Hagberg et al. 2004). PARP-1-mediated cell death has previously been explained in terms of NAD⁺ consumption and mitochondrial energy failure (Eliasson et al. 1998). A recent study suggests that PARP-1 mediate the release of AIF from mitochondria, resulting in caspase-independent cell death, a process which could be blocked by microinjection of an antibody against AIF (Yu et al. 2002). In

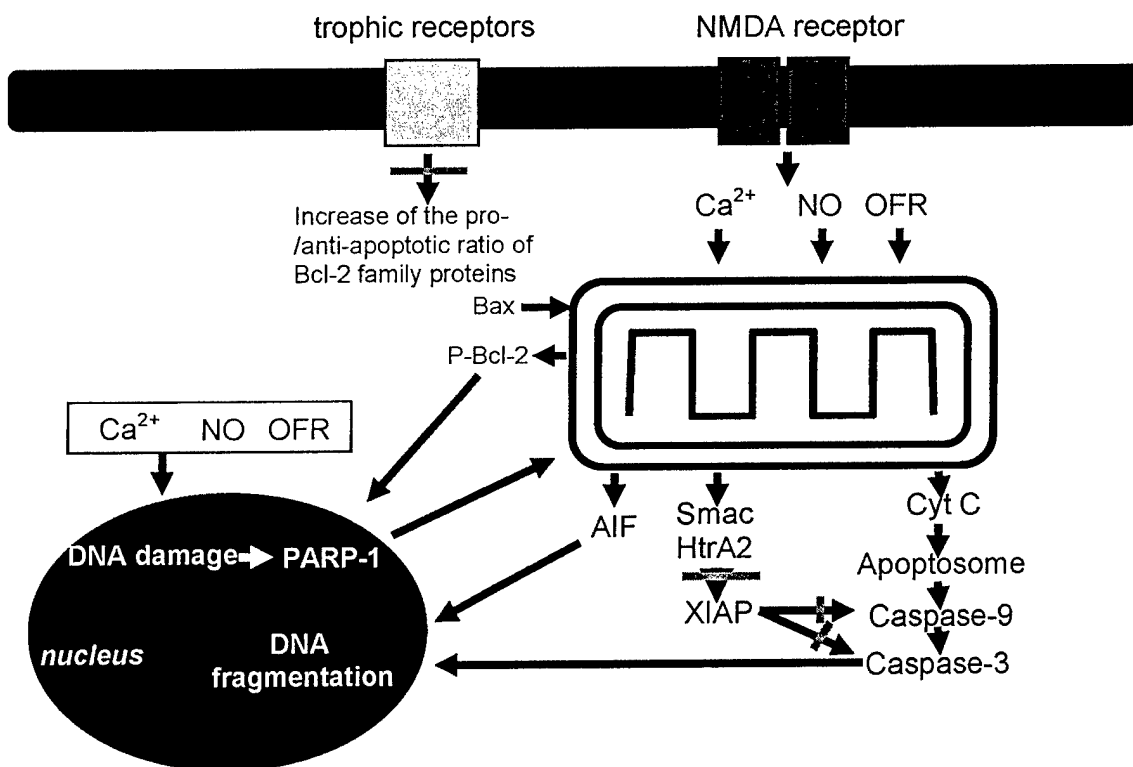


support of this hypothesis, we found that cells with increased PAR immunoreactivity after HI (indicative of activation of PARP-1) often exhibit a shift in AIF immunoreactivity from mitochondria to the nucleus. Irrespective of which molecular mechanisms that prove to be most important, it seems likely that mitochondria are important in PARP-1-mediated cell death (Yu et al. 2002,2003).

NMDA-receptor activation, nitric oxide (NO) and mitochondrial impairment

There are also other potential adverse factors in the mitochondrial environment that could be important (Figure). NMDA receptor activation with increased Ca^{2+} influx, free radical formation and induction of NO could damage mitochondrial membranes (Nowicki et al. 1982; Zaidan and Sims 1994; Schulz et al. 1995; Crow and Beckman, 1995). As previously mentioned, administration of NMDA receptor antagonists prevents the depression of mitochondrial respiration *in vivo* (Gilland and Hagberg, 1996). In addition, we have found that both NMDA receptor blockers (Puka-Sundvall, 2000c) and a combined inhibitor of inducible and neuronal NO synthase (2-iminobiotin) (Peeters-Scholte, 2002) inhibit activation of caspase-3, DNA-fragmentation and brain injury (Gilland and Hagberg, 1997). These data suggest indirectly that glutamate activation of NMDA receptors and production of NO may contribute to the mitochondrial release of pro-apoptotic proteins and subsequent activation of caspase-3.

In conclusion, neonatal HI induces activation of NMDA receptors, production of NO/oxygen free radicals, a loss of trophic factor support, speculatively leading to an increase in the ratio of pro-apoptotic/ anti-apoptotic Bcl-2 family proteins. The changed environment will lead to depression of mitochondrial respiration, intra-mitochondrial Ca^{2+} accumulation and swelling, mitochondrial permeability transition and release of pro-apoptotic proteins resulting in caspase-dependent and caspase-nondependent cell death (Figure).





Figure

Tentative role of mitochondria in cellular injury after HI in the developing CNS. AIF, Apoptosis-inducing factor; Bax, Bcl-2-associated protein X; Bcl-2, B-cell leukaemia gene 2; CytC, cytochrome C; Htr/A2, high-temperature requirement serine protease A2; OFR, oxygen free radicals; Smac, second mitochondrial activator of caspase; XIAP, X-linked inhibitor of apoptosis;

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MPTP as a Mitochondrial Neurotoxic Model of Parkinson's Disease

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1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent neurotoxin extensively used to model Parkinson's disease (PD). A cascade of deleterious events, in which mitochondria play a pivotal role, drives MPTP neurotoxicity. How mitochondria are affected by MPTP and how their defect contributes to the demise of dopaminergic neurons in this model of PD is discussed in this review.

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects (Ziering et al., 1947). MPTP can induce a parkinsonian syndrome in humans and non-human primates almost indistinguishable from Parkinson's disease (PD) on both clinical and neuropathological standpoints (Langston and Irwin, 1986). Over the years, MPTP has been used in a host of different animal species, especially in mice (Heikkila et al., 1989), to recapitulate the hallmark of PD cellular pathology, namely the degeneration of the nigrostriatal dopaminergic pathway (Dauer and Przedborski, 2003). Although the MPTP model departs from PD on several significant aspects, it continues to be regarded as the best experimental model of this common neurodegenerative disease. With respect to PD, enthusiasm for the MPTP model is driven by the belief that unraveling the MPTP neurotoxic process in animals may provide hints into the mechanisms responsible for the demise of dopaminergic neurons in human PD.

Various key cellular and molecular components underlying the MPTP neurotoxic process have been reviewed in details in the following references (Dauer and Przedborski, 2003; Przedborski and Vila, 2003) and will thus not be discussed here. Instead, the focus of this mini-review will be devoted to the role of the mitochondria in the deleterious effects of the parkinsonian toxin MPTP.

FIRST STEP FIRST

MPTP is a pro-toxin whose toxicokinetics is a complex, multistep process (Dauer and Przedborski, 2003). As indicated by its octanol/water partition coefficient of 15.6 (Riachi et al., 1989), MPTP is a highly lipophilic molecule, which is able to readily permeate lipid bilayer membranes. It is therefore not surprising to observe that MPTP crosses the blood-brain barrier in a matter of minutes after its systemic administration (Markey et al., 1984). Once in the brain, it is rapidly converted into 1-methyl-4-phenylpyridinium (MPP⁺), the actual neurotoxin (Heikkila et al., 1984). This critical transformation of MPTP into MPP⁺ is a two step process. First, MPTP undergoes a two electron oxidation, catalyzed by monoamine oxidase B (MAO-B), yielding the intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) (Chiba et al., 1984). Given the discrete cellular distribution of MAO-B in the brain (Kitahama et al., 1991), it is believed that the conversion of MPTP to MPDP⁺ occurs specifically in glial and serotonergic cells, and not in dopaminergic neurons. MPDP⁺ is an unstable molecule which readily undergoes spontaneous disproportionation to MPP⁺ and MPTP (Chiba et al., 1985; Peterson et al., 1985).

Once formed, MPP⁺ is presumably released from glial and serotonergic cells into the extracellular space prior to entering dopaminergic neurons. Yet, MPP⁺ has an octanol/water partition coefficient of 0.09 (Riachi et al., 1989), which indicates that, while being a lipophilic cation, MPP⁺ is far less lipophilic than MPTP. Thus, unlike MPTP, MPP⁺ is most likely unable to easily diffuse across cellular lipid bilayer membranes. Instead, it is to be expected that the release of MPP⁺ from its intracellular sites of formation and entry into adjacent neurons depend on specialized



carriers. Consistent with this view is the fact that MPP⁺ access to dopaminergic neurons relies on the plasma membrane dopamine transporter (Javitch et al., 1985; Bezard et al., 1999).

MITOCHONDRIAL ACCUMULATION

Once inside neurons, MPP⁺ rapidly accumulates in the mitochondrial matrix (Ramsay et al., 1986; Ramsay and Singer, 1986). Initially, it was thought that MPP⁺ gains access to the mitochondrial matrix through a carrier (Ramsay et al., 1986; Ramsay and Singer, 1986). However, it is now well established that MPP⁺ is passively transported (Hoppel et al., 1987; Davey et al., 1992) by a mechanism relying entirely upon the large mitochondrial transmembrane potential gradient ($\Delta\Psi$) of -150 to -170 mV (Ramsay et al., 1986; Ramsay and Singer, 1986; Hoppel et al., 1987; Davey et al., 1992; Aiuchi et al., 1988).

Like with other lipophilic cations (Rottenberg, 1984), the higher the concentrations of intramitochondrial MPP⁺, the lower the $\Delta\Psi$ and, consequently, the slower the uptake of extramitochondrial MPP⁺ (Hoppel et al., 1987; Davey et al., 1992). The demonstration that the ion-pairing agent tetraphenylboron anion increases both the rate and the extent of MPP⁺ uptake in isolated mitochondria (Hoppel et al., 1987; Davey et al., 1992; Aiuchi et al., 1988) further supports this concept. As discussed below, MPP⁺ inhibits mitochondrial respiration, which likely also contributes to the loss of the $\Delta\Psi$ gradient and to the dampening of the mitochondrial uptake of MPP⁺. It is thus not surprising that the accumulation of MPP⁺ by energized mitochondria behaves as a saturable phenomenon in the presence of high extramitochondrial concentrations of MPP⁺ (e.g., >10 mM) (Ramsay and Singer, 1986) and appears to reach a steady-state after a few minutes (Ramsay et al., 1986; Davey et al., 1992). This apparent steady-state persists until mitochondrial suspension becomes anaerobic or that the $\Delta\Psi$ is collapsed by the addition of an uncoupler agent such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Ramsay et al., 1986). Remarkably, energized mitochondria incubated with 0.5 mM MPP⁺ reach matrix concentrations of more than 24 mM after only 10 minutes (Ramsay and Singer, 1986). This fast and avid uptake suggests that most, if not all, of the cytosolic MPP⁺ would eventually accumulate in the mitochondrial matrix after the systemic injection of MPTP.

INTRAMITOCHONDRIAL MPP⁺

It is well established that intramitochondrial MPP⁺ inhibits oxidative phosphorylation (Nicklas et al., 1985; Singer et al., 1987). Intramitochondrial MPP⁺ also appears to inhibit the tricarboxylic acid cycle enzyme α -ketoglutarate dehydrogenase (Mizuno et al., 1987a). Although both mitochondrial metabolic alterations may contribute to MPP⁺ cytotoxicity, attention has been paid almost exclusively to the action of MPP⁺ on the respiratory chain.

It is well documented that MPP⁺ impairs, in a dose- and time-dependent manner, the ADP-stimulated oxygen consumption (State 3) in intact mitochondria supported by the NADH-linked substrates glutamate and malate (Nicklas et al., 1985; Mizuno et al., 1987b). MPP⁺ is, however, ineffective in inhibiting the oxygen consumption in mitochondria supported by succinate (Nicklas et al., 1985; Mizuno et al., 1987b). Furthermore, MPP⁺ prevents the binding of the classical Complex I inhibitor [¹⁴C]-rotenone to electron transport particles (Ramsay et al., 1991a). Collectively these findings indicate that MPP⁺, like rotenone and piericidin A, impairs mitochondrial respiration by inhibiting the multisubunit enzyme Complex I (i.e., NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain. This straightforward interpretation is supported by the electron spin resonance demonstration that MPP⁺ does actually bind to Complex I and blocks the terminal step of electron transfer from the highest potential iron-sulfur cluster of Complex I called N2 to ubiquinone (Ramsay et al., 1987).

The use of several MPP⁺ analogs and cationic inhibitors has demonstrated that MPP⁺ binds at two distinct sites within the mitochondrial electron transport chain region comprised between N2 and ubiquinone (Gluck et al., 1994; Ramsay et al., 1989; Ramsay et al., 1991b; Ramsay and Singer,



1992; Miyoshi et al., 1997; Miyoshi et al., 1998). These studies have also demonstrated that the occupation of both sites appears to be required for complete inhibition of NADH oxidation. The binding of MPP^+ to the first, more hydrophilic site seems to primarily affect the functional coupling between the PSST and the ND1 subunit of Complex I and to account for only 40% of the MPP^+ -induced reduction in NADH oxidation (Schuler and Casida, 2001). The binding of MPP^+ to the second, more hydrophobic site seems quite potent in blocking Complex I enzymatic activity (Schuler and Casida, 2001). Yet, the exact location of this second binding site in Complex I remains to be determined. Nonetheless, the importance of the binding to PSST, but not to the ND1 subunit in the inhibition of Complex I-mediated NADH oxidation (Schuler and Casida, 2001; Schuler et al., 1999) suggests that the MPP^+ hydrophobic site must also be situated somewhere in the PSST subunit. This hydrophobic site appears not to exist for other typical Complex I inhibitors such as rotenone and piericidin A (Schuler and Casida, 2001). Accordingly, while MPP^+ binds to Complex I, as do rotenone and piericidin A (Krueger et al., 1993; Gluck et al., 1994), it may not bind to exactly the same Complex I subunit or subunit part as these two other Complex I inhibitors. Also worth noting is the fact that MPP^+ , compared to rotenone and piericidin A, is a far weaker inhibitor of Complex I, which may explain why millimolar concentrations of MPP^+ are needed to inhibit NADH-oxidation in electron transport particles (Hoppel et al., 1987).

CONSEQUENCES OF MPP^+ -INDUCED COMPLEX I INHIBITION

In response to MPP^+ binding to Complex I, the flow of electrons along the respiratory chain is hampered in both dose- and time-dependent manners (Nicklas et al., 1985; Vyas et al., 1986; Hasegawa et al., 1990). The importance of the inhibition of Complex I in the MPTP-induced neurotoxicity *in vivo* is supported by the demonstration that strategies aimed at stimulating oxidative phosphorylation via by-passing the blockade of Complex I not only improve mitochondrial respiration but also mitigate dopaminergic neurodegeneration in mice (Tieu et al., 2003).

The current hypothesis on MPTP cytotoxicity posits that one of the main contributors to cell death is the impaired synthesis of ATP resulting from the inhibition of Complex I by MPP^+ . Relevant to this view is the fact that MPP^+ indeed causes a rapid and profound depletion of cellular ATP levels in isolated hepatocytes (Di Monte et al., 1986), in brain synaptosomal preparations (Scotcher et al., 1990), and in whole mouse brain tissues (Chan et al., 1991). It appears, however, that Complex I activity should be reduced by more than 50% to cause significant ATP depletion in non-synaptic brain mitochondria (Davey and Clark, 1996). Furthermore, *in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (Chan et al., 1991). These facts argue against MPP^+ -related ATP deficits being the sole factor underlying MPTP-induced cell death.

Another consequence of Complex I inhibition by MPP^+ is an increased production of reactive oxygen species (ROS). It was shown that incubation of MPTP with brain mitochondria resulted in an oxygen-dependent formation of ROS (Rossetti et al., 1988). It was also shown that incubation of MPP^+ with bovine heart submitochondrial particles causes a production of superoxide radicals when MPP^+ is used at the concentrations expected to be found inside neurons after MPTP systemic administration (Hasegawa et al., 1990). In this study, the authors also demonstrate that the degree of Complex I inhibition is proportional to the amount of superoxide radical produced (Hasegawa et al., 1990). Because modulations of key mitochondrial ROS scavengers, such as manganese superoxide dismutase, affect MPTP-induced neurotoxicity in mice (Klivenyi et al., 1998; Andreassen et al., 2001), it is reasonable to assert that MPP^+ -related ROS production also contributes to MPTP-induced cell death.

CONCLUSION

As discussed above, ATP depletion and ROS overproduction appear to occur soon after MPTP injection, subjecting the intoxicated cells, early on, to an energy crisis and oxidative stress. However, the time-course of these perturbations reviewed in the following reference (Przedborski



and Vila, 2003) appears to correlate poorly with the time-course of neuronal death in vivo (Jackson-Lewis et al., 1995). What this meta-analysis is suggesting is that only a few neurons are probably succumbing to the early combined effects of ATP depletion and ROS overproduction. Instead, mounting evidence discussed in following references (Przedborski and Vila, 2003; Dauer and Przedborski, 2003) indicates that rather than killing the cells, alterations in ATP synthesis and ROS production are pivotal in triggering cell death-related molecular pathways which, once activated, rapidly lead to the demise of the intoxicated neurons.

Interestingly enough, among these latter molecular pathways, it appears that the mitochondrial-dependent programmed cell death machinery plays a critical role (Vila et al., 2001). As illustrated in figure 1, it is thus plausible that the death of neurons caused by MPTP results from a circular cascade of deleterious events starting at the mitochondria by the alteration of the oxidative phosphorylation and finishing also at the mitochondria by the activation of the programmed cell death machinery. Whether the whole circuit depicted above is entirely orchestrated at the level of the mitochondria or whether it also involves perturbations that arise in the cytosol (e.g., protein nitration, cyclooxygenase-2 induction) and the nucleus (e.g., DNA damage, PARP activation) of the intoxicated cells is the focus of several ongoing studies in our laboratory.

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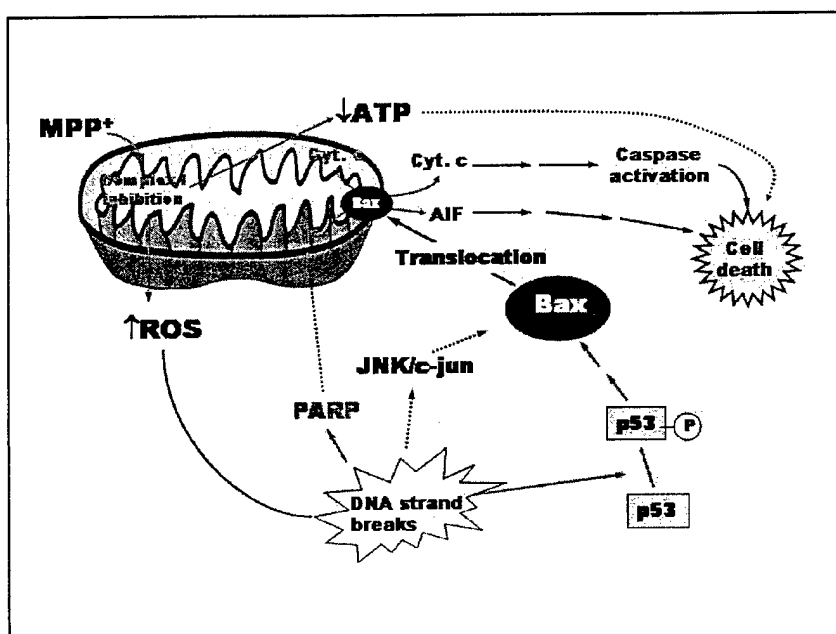


FIGURE LEGEND

Fig. 1. Illustration of the proposed circular nature of the MPP⁺-mediated cell death cascade. MPP⁺ enters in the mitochondrion and binds to Complex I, whereby it inhibits ATP synthesis and stimulates ROS production. These two initial events lead to a host of cellular perturbations such as DNA damage, which, in turn, trigger a variety of cell-death related pathways. These include activations of p53 by phosphorylation (p53-p) and JNK/c-Jun, which lead to Bax induction and translocation to the mitochondria. DNA damage also stimulates poly(ADP-ribose) polymerase (PARP) activity. Bax translocation and PARP activation promote the translocation of cytochrome c and apoptosis-inducing factor (AIF) from the mitochondria to the cytosol. Once in the cytosol, cytochrome C participates in a caspase-dependent cell death process, while AIF participates in a caspase-independent cell death process, both of which are not necessary mutually exclusive. Solid arrow, known mechanism; dashed-arrow, speculated mechanism.

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**Mitochondrial Dysfunction and Oxidative Damage in Alzheimer's and Parkinson's Disease
and Coenzyme Q₁₀ as a Potential Treatment**

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Abstract

There is substantial evidence that mitochondrial dysfunction and oxidative damage may play a key role in the pathogenesis of neurodegenerative diseases. Evidence supporting this in both Alzheimer's Disease and Parkinson's Disease is continuing to accumulate. The present review discusses the increasing evidence for a role of both mitochondrial dysfunction and oxidative damage in contributing to β -amyloid deposition in Alzheimer's Disease. We also discuss the increasing evidence that Parkinson's Disease is associated with abnormalities in the electron transport gene as well as oxidative damage. Lastly, we reviewed the potential efficacy of coenzyme Q as well as a number of other antioxidants in the treatment of both Parkinson's Disease and Alzheimer's Disease.

Alzheimer's Disease Transgenic Models: β -amyloid, energy metabolism and transgenic models

There is a large body of evidence implicating impaired energy metabolism and oxidative damage in the pathogenesis of AD. α -Ketoglutarate dehydrogenase complex activity is severely decreased in post-mortem AD brain [1]. This is unlikely simply to be secondary to cell loss, because the defect is also found in skin fibroblasts [2]. It however could be related vulnerability of the enzyme to oxidative damage. Genetic polymorphisms in one of the key components of α -ketoglutarate dehydrogenase dihydrolipoamide dehydrogenase is associated with AD [3]. A truncated gene product was recently found, which is localized to the intermembrane space of mitochondria. If expression of the truncated gene product is reduced, there is a marked decrease in amounts of subunits of complex I and IV of the mitochondrial electron transport chain and a decline of activity [3]. There is a link between mitochondrial abnormalities and oxidative stress in AD post-mortem tissue [4], and oxidative damage occurs early in the pathogenesis of AD [5]. Oxidative damage to lipids precedes β -amyloid deposition in a transgenic mouse model of AD [6].

There is also a large body of evidence implicating β -amyloid in the pathogenesis of AD. All genes thus far identified as causing AD are involved with the processing of β -amyloid. Trisomy 21 inevitably results in AD pathology [7], and the amyloid precursor protein (APP) gene is located on chromosome 21 [8]. Mutations in the APP gene result in early onset autosomal dominant AD [9, 10]. Mutations in presenilins, which also cause early onset autosomal dominant AD [11], increase levels of the particularly fibrillogenic species $A\beta_{42}$ [12, 13], through an effect on the γ -secretase [14, 15]. Finally, the $\epsilon 4$ allele of apolipoprotein E (apoE) increases the risk of late onset AD [16], and apoE4 binds directly to $A\beta$ and promotes its fibrillogenesis [17].

There are strong links between the mitochondrial and amyloid hypotheses. On one hand, mitochondrial dysfunction and oxidative stress may alter APP processing, leading to increased intracellular $A\beta$ accumulation. Inhibition of cytochrome oxidase results in accumulation of potentially amyloidogenic C-terminal fragments [18]. Free radical stress increases cellular $A\beta_{42}$ levels [19]. Uncoupling mitochondria with FCCP in normal astrocytes recapitulates the altered APP processing and intracellular accumulation of $A\beta_{42}$ seen in astrocytes and neuronal cultures from



fetal Down's syndrome brain [20]. There is also evidence that oxidative stress increases the activity of β -secretase, the enzyme responsible for N-terminal cleavage of β -amyloid from the amyloid precursor protein [21, 22]. In Down's syndrome, there is evidence that oxidative damage precedes β -amyloid deposition [23].

On the other hand, β -amyloid may cause mitochondrial dysfunction and oxidative stress. β -amyloid suppresses mitochondrial succinate dehydrogenase, and inhibits of PC12 cell redox activity [24, 25]. Exposure of isolated rat brain mitochondria to β -amyloid caused a significant reduction in state 3 and state 4 respiration [26]. β -amyloid protein induces oxidative damage to mitochondrial DNA in PC12 cells [27], and there is increased generation of reactive oxygen species in neurons cultured from fetal Down's syndrome [28]. Recently, a direct link between the mitochondrial and amyloid hypotheses was demonstrated, by showing that APP is physically targeted to mitochondria and impairs mitochondrial function in neuronal cells [29].

There are now several transgenic animal models which show increased β -amyloid deposition. These include transgenic mice overexpressing APP with the V717F mutation, and the Swedish double mutation at positions 670/671 (Tg2576) [30, 31, 32]. These mice are analogous to recently described mice. These TgCRND8 mice have a double mutant form of the amyloid precursor protein 695 (KM 670/671 NL and V717F), under control of the PrP gene promoter [33]. The mice show thioflavin S-positive β -amyloid deposits at 3 months of age, and dense-cored plaques and neuritic pathology from 5 months of age. In the Tg2576 mice, β -amyloid deposits are associated with evidence of oxidative stress as assessed by immunostaining [34, 35], and oxidative damage to lipids appears to precede β -amyloid deposition in AD transgenic mice [6].

A recent paper showed that intracellular accumulated β -amyloid precedes both neurofibrillary tangles and synaptic dysfunction in a transgenic mouse expressing β -amyloid, presenilin, and tau mutations [36]. We examined the effects of crossing mice with a partial deficiency of manganese superoxide dismutase with Tg1995 mice [37]. This markedly exacerbated β -amyloid deposition, providing direct evidence of a link between β -amyloid deposition and oxidative damage.

Mitochondrial Dysfunction in PD

The possible role of oxidative damage and mitochondrial dysfunction in PD has been strengthened by the finding that chronic infusions of the complex I inhibitor rotenone produce an animal model of PD in rats [38]. The infusions produced a selective loss of substantia nigra dopaminergic neurons as well as cytoplasmic α -synuclein immunoreactive inclusions closely resembling Lewy bodies. The mechanisms of neurotoxicity appears to involve oxidative damage [39].

Evidence for mitochondrial dysfunction of idiopathic PD comes from a 30-40% decrease in complex I activity in the substantia nigra [40, 41, 42, 43]. Reduced staining for complex I subunits in PD substantia nigra, but preserved staining for subunits of the other electron transport complexes has been demonstrated immunohistochemically [44]. Strong support for a mitochondrial DNA encoded defect comes from studies which showed that complex I defects from PD platelets are transferable into mitochondrial deficient cell lines [45, 46]. These defects are associated with increased free radical production, increased susceptibility to MPP⁺, and impaired mitochondrial calcium buffering [47]. Direct sequencing of mitochondrial complex I and tRNA genes failed to show homoplasmic mutations [48].

A number of other recent studies, however, provide genetic evidence that mitochondrial DNA abnormalities may contribute to PD pathogenesis. An out of frame cytochrome b gene deletion occurred in a patient with Parkinsonism was associated with increased free radical production [49]. A novel mitochondrial 12 SrRNA point mutation was found in a pedigree with Parkinsonism, deafness and neuropathy [50]. We found Parkinsonism occurred in association with



the Leber's optic atrophy mitochondrial mutation G11778A [51]. An increase in mitochondrial DNA deletions/rearrangements and novel complex I mutations were found in the substantia nigra of PD patients [52, 53]. Lastly mitochondrial haplotypes in caucasian patients (classified as haplotype J) markedly reduce the risk of developing PD [54]. The mRNA for the NDI subunit of mitochondrial complex I is reduced by 25% in the substantia nigra melanized neurons in PD [55].

Oxidative Damage in PD

A great deal of interest has focused on the possibility that oxidative damage may play a role in the pathogenesis of PD. There are studies showing increased levels of malondialdehyde and cholesterol lipid hydroperoxides, markers for lipid peroxidation, in PD substantia nigra [56, 57]. There are widespread increases in protein carbonyls in PD postmortem brain tissue [58]. Concentrations of 8-hydroxy-2-deoxyguanosine, a marker of oxidative damage to DNA, are significantly increased in PD substantia nigra and striatum [59, 58, 60]. There is evidence for nitrosyl radicals in PD substantia nigra [61]. Another means of looking for oxidative stress is to measure concentrations of reduced glutathione. Reduced glutathione is decreased in PD substantia nigra by approximately 50% [62, 63, 64, 65]. Individuals with incidental Lewy body disease may have presymptomatic PD, and they have a 35% reduction in reduced glutathione as compared with age-matched controls [66].

Other studies showed an increase in oxidative damage to cytoplasmic DNA and RNA in substantia nigra in PD as detected using immunocytochemistry [60]. An increase in oxidative damage to DNA was also reported in leukocytes, serum and CSF of PD patients [67,68]. An increase in 3-nitrotyrosine immunoreactivity was reported in Lewy bodies in PD [69]. This finding was confirmed with antibodies specific for nitrated α -synuclein [70]. This finding provides a link between oxidative damage and protein aggregates, which are characteristic features of PD. Strengthening this is the observation that intracellular production of peroxynitrite induces α -synuclein aggregation [71]. Other evidence shows that oxidative damage impairs ubiquitination and degradation of proteins by the proteasome [72].

Coenzyme Q₁₀ and Neuroprotection

There is increasing interest in the potential usefulness of coenzyme Q₁₀ (CoQ₁₀) to treat neurodegenerative diseases. CoQ₁₀ serves as an important co-factor of the electron transport chain, where it accepts electrons from complexes I and II [73, 74]. CoQ₁₀, which is also known as ubiquinone, serves as an important antioxidant in both mitochondria and lipid membranes. It mediates some of its antioxidant effects through interactions with alpha-tocopherol [73, 75]. Coenzyme Q₁₀ blocks apoptosis by inhibiting activation of the mitochondrial permeability transition independently of its free radical scavenging activity [76]. Another potential neuroprotective mechanism of coenzyme Q₁₀ is as a cofactor of mitochondrial uncoupling proteins [77, 78]. Coenzyme Q₁₀ is also an obligatory co-factor for mitochondrial uncoupling proteins [78, 77]. Activation of these proteins reduces mitochondrial free radical generation. Coenzyme Q induces mitochondrial uncoupling in the substantia nigra of primates, and this is associated with marked neuroprotection against MPTP toxicity [79]. Increased expression of mitochondrial uncoupling proteins protects against brain damage associated with both experimental stroke and epilepsy [80, 81].

CoQ₁₀ diminished ischemia induced neuronal injury in the hippocampus [82]. CoQ₁₀ protects cultured cerebellar neurons against excitotoxin-induced degeneration [83]. We studied the effects of administration of CoQ₁₀ on lesions produced by mitochondrial toxins. Oral administration of CoQ₁₀ produced dose-dependent neuroprotective effects against malonate induced striatal lesions as well as depletions of ATP and increases in lactate concentrations [84]. Administration of CoQ₁₀ produced significant protection against dopamine depletions induced by MPTP administration [85]. Oral administration of CoQ₁₀ for one week prior to co-administration of 3-nitropropionic acid resulted



in a significant 90% neuroprotection against 3-nitropropionic acid induced striatal lesions [86]. We found that oral administration of CoQ₁₀ starting at 50 days of age significantly increased life span of ALS transgenic mice [86], and increased survival in HD transgenic mice by 14.5 % [87]. Administration of CoQ₁₀ significantly delayed the development of motor deficits, weight loss, cerebral atrophy and neuronal inclusions.

We administered CoQ₁₀ at a dose of 360 mg per day to HD patients for 1 to 2 months [88]. CoQ₁₀ therapy led to a significant 37% reduction in occipital cortex lactate concentrations, which reversed following discontinuation of therapy, indicating a therapeutic effect of CoQ₁₀. A tolerability study of CoQ₁₀ in HD patients showed that there were minimal adverse effects at doses of 600 to 1200 mg daily [89]. In the CARE-HD trial 360 patients were treated for 30 months [90]. They were randomized to CoQ₁₀ at 600 mg per day, remacemide at 600 mg per day or the combination in a 2 x 2 factorial design. The primary outcome variable was changed in the Unified Huntington's Disease Rating Scale. In this trial, CoQ₁₀ slowed decline on the total functional capacity measure scale by 14% over the 30 months.

The CoQ analogue idebenone reduces cardiac hypertrophy in Friedreich's Ataxia patients [91, 92]. A study of the effects of CoQ₁₀ in Friedreich's ataxia patients showed improvement of cardiac and skeletal muscle bioenergetics [93]. Coenzyme Q₁₀ was administered at 400 mg daily and after 3 months of treatment the cardiac phosphocreatine to ATP ratios showed a mean relative increase to 178% of initial values.

A phase-II clinical trial in Parkinson's Disease patients enrolled 80 patients who were randomly assigned to placebo or CoQ₁₀ at doses of 300, 600 or 1200 mg per day [94]. The primary outcome measure was the Unified Parkinson's Disease Rating Scale (UPDRS) which was administered at screening, baseline, and 1, 4, 8, 12 and 16 months. The subjects were patients with early PD who did not require treatment (levodopa) for their disability. They were followed up for 16 months or until disability requiring treatment with levodopa had developed. The difference between the 1200 mg and placebo groups was significant with a $p = 0.04$, with an overall slowing of disability of 44% at 16 months.

Antioxidants and AD

A prior study showed that vitamin E has efficacy in slowing the progression of AD [95]. Ginkgo biloba also may exert beneficial effects [96, 97]. The antioxidants curcumin and melatonin exert beneficial effects on amyloid deposition in transgenic mouse models of AD [98, 99]. It is, therefore, possible that CoQ₁₀ might similarly be beneficial in AD.

Conclusions

There is a large body of evidence implicating both mitochondrial dysfunction and oxidative damage in the pathogenesis of AD and PD. CoQ₁₀ administration can increase total brain levels of CoQ₁₀ and brain mitochondrial CoQ₁₀ levels in mature and older animals. There is substantial evidence that CoQ₁₀ can act in concert with alpha-tocopherol as an antioxidant within mitochondria. CoQ₁₀ administration is neuroprotective against ischemia and lesions produced by mitochondrial toxins including malonate, 3-nitropropionic acid and MPTP. CoQ₁₀ extends survival in a transgenic mouse models of ALS and HD. Initial clinical trials in Friedreich's ataxia, HD and PD have shown beneficial effects. Several other antioxidants have the potential of ameliorating the progressive neurodegeneration which occurs in AD and PD. Lastly, it is possible that antioxidants may have additive or synergistic effects with agents targeting other modalities of cell death, such as apoptosis.

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Development of Mitochondrial Gene Replacement Therapy

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Known point mutations in mitochondrial DNA (mtDNA) are relatively rare and associated with a wide variety of "mitochondrial" diseases affecting brain, retina, optic nerve, muscle, heart, endocrine organs and liver¹⁻⁵. These conditions are notable for delayed expression of variable phenotypes, and the underlying mechanisms of cellular pathophysiology remain unclear. Because the mitochondrial genome codes for only 13 out of the ~85 electron transport proteins and the hundreds or probably >1000 of all mitochondrial proteins, it remains challenging to formulate how one or more mutations in this small genome can have such profound physiological effects. In addition, mtDNA deletions accumulate with aging and may contribute to bioenergetic failure of older muscle fibers and neurons, resulting in sarcopenia and degenerative diseases such as Alzheimer's and Parkinson's⁶. In all of these conditions, understanding the dynamics of mitochondrial genome replication and expression in individual cells will provide insight into disease pathophysiology. For those conditions where mitochondrial genome mutations are causal for disease expression, supplementation with normal mitochondrial genomes, or ideally replacement of defective with normal mitochondrial genomes, has great therapeutic potential. However, carrying out these critical studies has been hampered by limitations in manipulating *in situ* the mitochondrial genome inside mitochondria of living cells.

We have recently developed novel technologies to remove and replace the human mitochondrial genome inside mitochondria of human cells. Using lambda phage virus as a transfection vector and lambdaphage receptor targeted to mitochondria, we demonstrated that the entire human mitochondrial genome with an inserted mitochondrial-specific GFP reporter can be transfected into rho⁰ mitochondria (mitochondria without any mtDNA) of cells. In this process ("mitofection"), mtDNA replication, mitochondrial GFP expression and restoration of bioenergetic function occur rapidly over several days (Khan, et al, under review). Subsequently, the original "mitofection" technology has been significantly improved by the development of an engineered protein transduction system to transport mtDNA across cell membranes and target it to mitochondria. This technology ("protofection") introduces mtDNA into mitochondria within minutes, restores bioenergetic activity of rho⁰ cells within 1-2 days, and is active *in vivo* in animals. Protofection can be used to deliver the entire normal mitochondrial genome, or PCR-generated fragments, mutations or deletions. Third, silencing the mtDNA polymerase (POL- γ) by RNA-interference results in complete loss of detectable POL- γ activity and detectable mtDNA within 72 hours with recovery of activity in 5-7 days. This allows creation of rho⁰ cells quickly without the use of mutagens or reverse transcriptase inhibitors and is applicable to non-dividing cells such as neurons. The combined use of RNA-interference to silence genes critical for mtDNA replication, and "protofection" to introduce healthy mitochondrial genomes into mitochondria of living cells sets the stage for the realistic possibility for mitochondrial gene therapy of a wide variety of conditions.

A general introduction to mitochondria and their genes

Although the origins of modern mitochondria are not known with certainty, the endosymbiotic theory proposed originally by Margulis⁷ remains one of the most cogent. In this construct, modern mitochondria developed from bacterial precursors who inhabited early prokaryotes and provided a mechanism to detoxify oxygen. These bacterial invaders established a complex symbiotic



relationship with their hosts that included improved metabolic efficiency and sharing of genomic responsibilities, with gradual transfer of mitochondrial genomic responsibilities to the host nucleus⁸. Early in evolution, the mitochondrial genome of eukaryotes thus shrank from its large bacterial predecessor to a much reduced size (366.9 kB in *A. thaliana*; 85.8 kB in *S. cerevisiae*; 13.8 kB in *C. elegans*; 16.5 kB in *H. sapiens*) The human mitochondrial genome, similar to that of other mammals, is intron-less, circular and codes for 13 electron transport proteins, 2 ribosomal RNA's and 22 tRNA's. The mitochondrial genetic code (m) is similar to but not identical with the nuclear code (n), differing in four codons (*AUA*=Ile (n), Met (m); *UGA*=Term (n), Trp (m); *AGA*, *AGG*=Arg (n), Term (m)).

The total number of mitochondrial proteins is not accurately known, but certainly numbers in the hundreds and possibly thousands^{9,10}. The vast majority of mammalian mitochondrial proteins are thus coded by nuclear genes and targeted to mitochondria by N-terminal mitochondrial localization sequences (MLS). The MLS-targeted proteins are imported into mitochondria in an energy-dependent manner by membrane translocase complexes, known as the translocase of outer membrane (TOM) and translocase of inner membrane (TIM). Following importation, the MLS is removed and proteins incorporated into electron transport chain, outer or inner membrane, intermembrane space or matrix.

The myriads of critical functions performed by mitochondria, including both the historically first described role of respiration and ATP synthesis, and now including the participation in calcium signaling and buffering and control of cell death activation¹¹⁻¹⁷, require coordinated expression and stoichiometrically regulated importation and incorporation of the hundreds (at least) of nuclear genome-encoded proteins of diverse functions and expression of 13 mitochondrial genome-encoded proteins devoted to electron transport chain function. Considering the complexity of this critical organelle, it is remarkable that it consistently is assembled in working order, is repaired efficiently and is passed on regularly to progeny cells.

Transduction Domains for Delivery of Therapeutic Proteins

The blossoming field of genomics, through utilization of advanced transfection protocols and gene microarrays, is leading researchers to the discovery of many novel therapeutic proteins. However, delivery of these large proteins across cell membranes, into senescent cells and across the blood-brain barrier (BBB) presents a substantial hurdle to utilizing this method of therapeutic intervention. With the introduction of Protein Transduction Domain (PTD)-Protein fusions, the hurdle is diminishing and appears to be increasingly surmountable. These small regions of proteins are able to cross the cell membrane in a receptor-independent mechanism.

Although several of these PTD's have been documented, the two most commonly employed PTDs are derived from the TAT protein from HIV and Antennapedia transcription factor from *Drosophila*, whose PTD is known as Penetratin¹⁸.

TAT protein consists of 86 amino acids and is involved in the replication of HIV-1. The TAT PTD consists of an 11 amino acid sequence domain of the parent protein that appears to be critical for uptake¹⁹. In the current literature TAT has been favored for fusion to proteins of interest for cellular import. Several modifications to TAT, including substitutions of glutamine to alanine (Q→A), have also demonstrated an increase in cellular uptake anywhere from 90%²⁰ to up to 33 fold in mammalian cells²¹.

Properties of Protein Transduction Domains

Highly efficient uptake

Intracellular delivery of various therapeutic proteins involving TAT-PTD fusions has proven to be quite effective. This type of fusion protein was recently utilized in the delivery of biologically



active antioxidant enzymes such as catalase (CAT). When exposed to H_2O_2 , HeLa cells demonstrated a 90% increase in cell viability as compared to controls²².

Kinetic studies on the uptake of PTD have shown that an entire cell population can reach maximum uptake of the PTD within as little as 30 seconds to 5 minutes of initial exposure²¹. PTDs provide for rapid uptake of attached proteins, although these fusion proteins can vary in uptake in a tissue specific manner and also depend on the structure and size of the protein fused.

Stability of transduced fusion proteins into cultured HeLa cells demonstrated a peak concentration at approximately two hours of incubation with a steady decrease up to seventy-two hours later²². Tat-PTD has also been fused to Angiotensin II type I receptor (AT_1R) to investigate Tat-PTD fusion's transduction efficacy and functionality in neurons. Neuronal cultures isolated from the hypothalamus and brainstem of 1 day old Wistar-Kyoto rats (WKY) were incubated with 300ug/ML of the recombinant protein and peak fluorescence was noted after 30 minutes of incubation with initial fluorescence recorded within minutes²³. These are just a few of the many examples of PTD linked proteins that demonstrate the ability of PTD to rapidly transduce cells.

PTD Fusion Proteins allow delivery of large cargo across the Blood Brain Barrier

Viral mediated delivery of DNA for the production of proteins is a potentially promising technology, but it is not well suited for certain conditions, as the delivery of genes via viral vector systems is time-consuming and often presents problems of immunogenicity. Protein synthesis can also be down regulated in areas of the brain which have undergone insult, such as ischemia, as well as having undergone pathophysiological change, as is seen in MELAS brain.

Another problem with the therapeutic delivery of proteins to neuronal tissues is the blood-brain barrier (BBB). The BBB is composed of specialized endothelial cells and tight junctions, which make delivery of even low molecular weight proteins, such as NGF (26 kDa dimer), a very difficult and low efficiency process.

Protein transduction domains present a new and exciting approach to the delivery of biologically active proteins across the BBB. Kilic et.al²⁴ recently demonstrated the ability of a Tat-GDNF (Glial cell line-derived neurotrophic factor) protein to cross the BBB. Delivery of the Tat-GDNF fusion prevented both apoptotic and necrotic injury after short and long term ischemia in rats. The method of application for the Tat-GDNF recombinant protein was intravenous infusion--requiring no surgical interventions.

Cao et al.²⁵ further demonstrated the ability of PTD proteins to cross the blood brain barrier utilizing a Bcl-xL PTD fusion. The aim of this study was to introduce Bcl-XI, a known neuronal anti-apoptotic factor, to provide neuroprotection during ischemia in the murine model of focal ischemia/reperfusion. Intraperitoneal injection of PTD-HA-Bcl-xL into mice demonstrated the ability of fusion proteins to cross the blood brain barrier. The protein fusion was able to decrease cerebral infarction up to 40% upon initiation of cerebral ischemia²⁵.

A similar study utilized a Bcl-x mutant (FNK), with increased anti-apoptotic activity, to protect SH-SY5Y neuroblastoma cells in vitro when exposed to staurosporine-induced apoptosis and glutamate-induced excitotoxicity. This PTD-FNK fusion was also injected i.p. into gerbils and prevented delayed neuronal death in the hippocampus caused by transient global ischemia.²⁶

Cytotoxicity and Immunogenicity

A key requirement for any therapeutic intervention with a PTD fusion protein is that no untoward changes in normal cell physiology or function occur. Brain microvascular endothelial cells (BMEC) exposed to Tat demonstrate marked increased levels of cellular oxidative stress, decreased levels of intracellular glutathione and activated DNA binding activity and transactivation of NF-kappaB and AP-1²⁷.

The protein transduction domain utilized by us is an 11 amino acid sequence that represents a poly-Arginine stretch shown to be higher in transduction efficiency than the PTD of the Tat-HIV-1



protein. Although the 11 amino acid PTD sequence is similar to the small motif of the parent Tat-HIV-1 protein, the concern that it may be sufficient to elicit similar cytotoxicity when introduced into cell culture or animal models is noteworthy and has been addressed in numerous publications since the discovery of PTD's. The literature to date indicates that the Tat-PTD can transduce proteins of interest to nearly 100% of a cell population without exhibiting cytotoxic effects. Many groups, such as Cao et al (discussed in capability of PTD to cross BBB), have also gone so far to prove the therapeutic benefits and the cell-protective capabilities that PTD-linked proteins possess. No cytotoxicity was reported upon treatment with the PTD fusion.²⁸ Jin et al²² utilized a Tat PTD-linked SOD (super oxide dismutase) and a Tat PTD-linked hCat (human catalase) to demonstrate that the transduced fusion proteins remained enzymatically stable for 60 h. The fusion protein did not elicit any cellular toxicity, and was able to increase HeLa cell viability up to 90% upon exposure to H₂O₂. Leifert et al²⁹ also recently reported that full-length proteins attached to the HIV Tat protein transduction domain are neither transduced between cells, nor exhibit enhanced immunogenicity. These experiments, as well as many others in the literature to date, demonstrate the potential therapeutic efficacy of PTD-linked proteins with no found toxicity or increased immunogenicity of the fusion proteins.

Mitochondrial Localization of PTD-Fusion Proteins

Del Gaizo, et al have characterized the feasibility of using protein transduction to target proteins to mitochondria. They found that a mitochondrial localization signal was necessary to enable persistence of the targeted protein inside mitochondria. Also, neither loss of the mitochondrial import machinery nor decrease in $\square\square_M$ inhibited entry and retention of their fusion protein. Finally, in pregnant mice injected with their TAT-mMDH-GFP fusion protein, the protein crossed the placenta and was found in fetal and neonatal pups, indicating that the protein not only crossed multiple membrane barriers but also persisted within mitochondria.^{30,31}

TFAM is a mitochondrial histone

Mitochondrial transcription factor A (TFAM) is a 246 amino acid (~25 kDa) protein first isolated and cloned as a transcription factor for mtDNA³². It is a member of the High Mobility Group (HMG) of proteins, contains two HMG domains and a 42 amino acid mitochondrial localization sequence, binds to ~25 bp of mtDNA and is capable of bending and unwinding mtDNA^{33,34}. Several important TFAM binding regions on mtDNA have been identified, and endogenous mtDNA is bound to ~1000-fold molecular excess of TFAM³⁵. TFAM is critical for mtDNA replication³⁶ and is controlled by transcription factors such as NRF-1 and NRF-2 known to regulate mitochondrial biogenesis³⁷.

Figure 1 shows the plasmid construction for creation of the recombinant PTD-MLS-TFAM and the resulting molecule. Figure 2 shows successful introduction into and replication of LHON mtDNA in rho⁰ cells. The LHON 11778A mutation causes loss of the SfaN1 site present in w.t. mtDNA. In rho⁰ cells a similar w.t.-like pseudogene is amplified and cut by SfaN1. Following protfection of LHON mtDNA into rho⁰ and passage through metabolic selection, mainly the introduced LHON mtDNA free of the SfaN1 site is found.

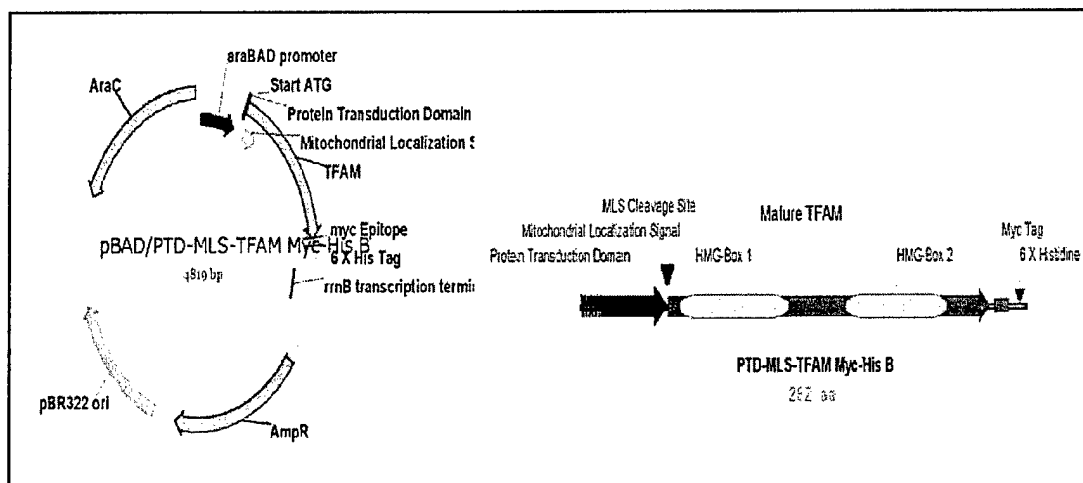


Figure 1. Plasmid design (left) and protein structure (right) for TFAM with a PTD domain followed by a MLS.

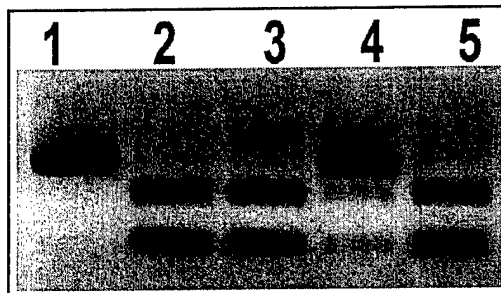


Figure 2. Agarose gel of PCR products amplifying region around LHON 11778A mutation after SfaN1. Lane 1-LHON Cybrid; Lane 2-Sy5y; Lane 3-Rho⁰; Lane 4- LHON mtDNA Prototected Rho⁰; Lane 5- Prototected Rho⁰ no DNA

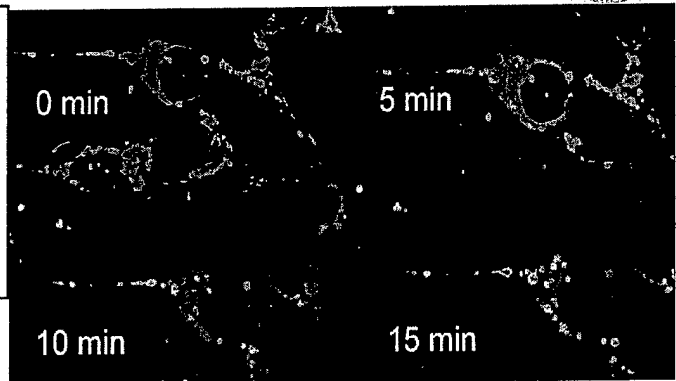


Figure 3. Timecourse of Alexa 488 Labeled MtDNA complexed with PTD-MLS-TFAM added to Sy5y cells. Red=MitoTracker Red

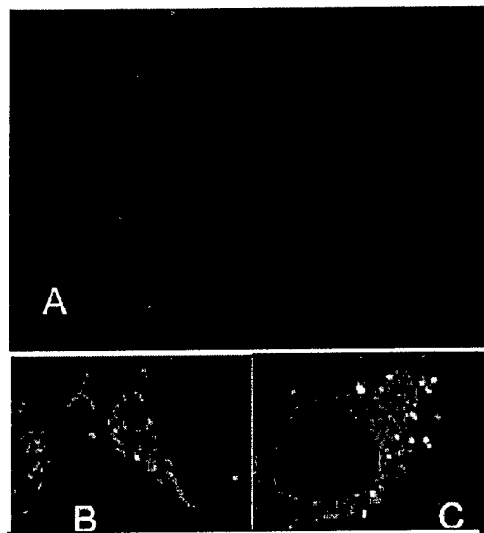


Figure 4. MtRed and BrdU (FITC) staining of rho⁰ (A), normal SY5Y (B) and rho⁰ 16 hrs after protfection with mtDNA complexed with PTD-MLS-TFAM (C).

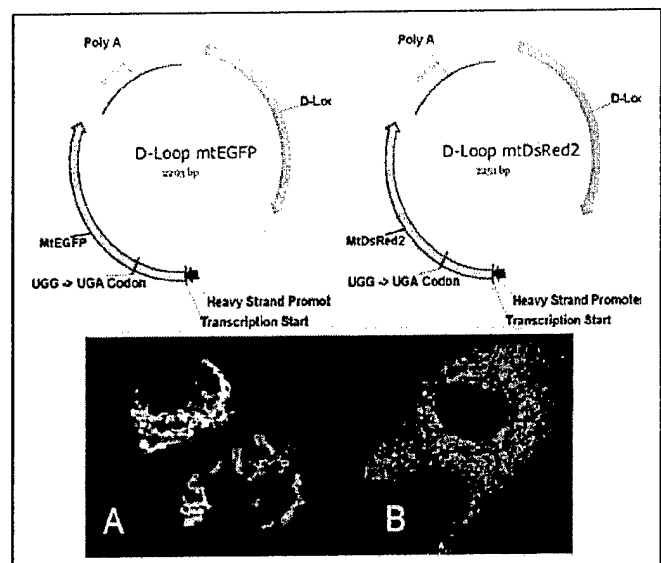


Figure 5. (top) Constructs for generating D-loop MtEGFP (left) and D-loop MtDsRed2 (right). (bottom). Normal SY5Y cell (A) and human cortical neuron (HCN, B) 24 hours after Protfection with D-loop MtEGFP construct and counterstained with MitoTracker Red.



Figure 3 shows that w.t. mtDNA that has been labeled with Alexa 488 dye and complexed with PTD-MLS-TFAM rapidly enters mitochondria of SY5Y cells and is concentrated within 15 minutes. Figure 4 shows the rapid restoration of mtDNA replication and bioenergetic function following introduction of w.t. mtDNA by protofection. The top image (A) is of ρ^0 cells stained with MitoTracker Red (MTRed), to localize mitochondria as a function of their $\square\square_M$, and following incubation for 12 hours with BrdU and immunostained for BrdU with FITC. Note the low levels of MTRed accumulation, reflecting low $\square\square_M$, and absence of BrdU staining. Part (B) shows a normal SY5Y cell and part (C) shows a ρ^0 cell 16 hours after protofection with PTD-MLS-TFAM complexed with w.t. mtDNA. Note the marked increase in MTRed uptake and BrdU staining.

Expression of exogenous genes in mitochondria using Protfection.

Because of our desire to be able to express individual genes inside mitochondria, in addition to the entire mitochondrial genome, we have pursued using Protfection technology to deliver small reporter genes directly to mitochondria. The premise behind the constructs is to place a gene of interest downstream of the mitochondrial D-loop and heavy strand promoter and incorporate a polyA tail. The constructs for D-loop-MtEGFP and D-loop-MtDsRed2 are shown in Figure 5. Recall that the two reporter fluorescent proteins have been mutated so as to be specific for the mitochondrial translation apparatus. Figure 5 also shows a normal SY5Y cell (A) and a human cortical neuron (B) 24 hours after Protfection with D-loop MtEGFP. There is robust mitochondrial GFP signal in mitochondria of the SY5Y and several small areas of EGFP signal in the human cortical neurons.

Protfection technology and mitochondrial gene therapy

The above results show that PTD-MLS-TFAM protfection technology provides a rapid and efficient approach to providing "healthy" mitochondrial genomes to cells. Theoretically, mitochondrial diseases with homoplasmic mutations can be treated by dilution of the pathogenic mitochondrial genomes with healthy ones. Exogenous individual genes of interest can also be introduced into mitochondria, and with the appropriate codon changes, can be restricted to mitochondrial translation.

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Mitochondrial Degeneration in ALS

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Introduction

Amyotrophic lateral sclerosis (ALS) is an age-dependent neurodegenerative disease that causes motor neuron degeneration, progressive skeletal muscle atrophy, paralysis and death (Rowland and Shneider, 2001). The etiology in most cases is not clear. Studies have implicated redox imbalance, protein aggregation, cytoskeleton disorganization, defective axonal transport and chronic ischemia playing a role in motor neuron death (Cleveland and Rothstein, 2001). Environmental toxins and lack of dietary vitamin E have also been suggested to cause this disease (De la Rua-Domenech *et al.*, 1997; Cox *et al.*, 2003).

One crucial question in understanding the disease mechanism is how these different noxious assaults lead to motor neuron degeneration. Are there common, converging cellular pathways? Early studies on humans were limited to pathological examination of autopsy specimens. Those studies revealed gigantic neurofilament swellings in proximal axons (Carpenter, 1968). For some years the neurofilament abnormalities had been the focus for studying the disease mechanism (Hirano, 1991).

In 1993, the first genetic cause for ALS was discovered (Rosen *et al.*, 1993). Mutations in Cu, Zn superoxide dismutase (SOD1) cause a subset of familial ALS. Soon after, several animal models for ALS were constructed by expressing the disease-associated SOD1 mutants in transgenic mice (Gurney, 1994; Ripps *et al.*, 1995; Wong *et al.*, 1995; Bruijn *et al.*, 1997). These transgenic mice have allowed studies of motor neuron degeneration process in unprecedented detail. It is from these studies that mitochondrial degeneration and dysfunction have emerged as an important early event in motor neuron degeneration process. This review summarizes efforts from our laboratory in characterizing mitochondrial degeneration in an ALS animal model. Readers who are interested in learning more about mitochondrial degeneration in ALS are referred to several broad reviews that are published recently (Julien, 2001; Menzies *et al.*, 2002a).

Mitochondrial degeneration is an early event in motor neuron degeneration pathway

Early observations on mutant SOD1 transgenic mice reported mitochondrial vacuolation as well as neurofilament accumulation in motor neurons (Dal Canto and Gurney, 1995; Wong *et al.*, 1995; Julien, 2001). The neurofilament pathology was expected but the severe mitochondrial abnormalities were a surprise. Were these pathologies revealing crucial steps in motor neuron degeneration pathway or were they simply representing byproducts of degenerative process—a mere consequence of cellular degeneration? This question motivated us to conduct a systematic study. We reasoned that abnormalities that occurred early in the disease process were more likely to contribute to the progression of the degeneration while the abnormalities that appeared late in the disease stages, when substantial neuronal death had already been well underway, were more likely to be a consequence of early degenerative process. The early events are also more interesting in terms of therapeutic intervention, because stopping disease progression early in its track enhances the probability of motor neurons being rescued. Thus, a study that correlates the



clinical progression and the sequence of pathological events (the kind of study that is impossible to conduct in humans) would particularly be revealing.

We conducted our studies in a low expresser line of mutant SOD1G93A that was generously made available by Gurney and colleagues (Gurney *et al.*, 1994). Because the onset and progression of the disease in the mutant SOD1 transgenic animals were heterogeneous, we first used a behavioral assay to determine the process of clinical disease progression. By this assay the disease progression was divided into four stages according to the relative muscle strength measured from the mice: a pre-muscle weakness (PMW) stage during which the muscle strength remained steady in mutant mice and was indistinguishable from wild type mice; a rapid declining (RD) stage during which the muscle strength declined suddenly and precipitously; a slow declining (SD) stage during which the muscle strength declined gradually in a prolonged period; and finally the paralysis stage during which one or multiple limbs became total immobile (Kong and Xu, 1998; Fig. 1). Interestingly, similar patterns of clinical progression, particularly the rapid decline of muscle strength at the onset of ALS, have also been reported in human longitudinal observations (Kasarskis and Winslow, 1989; Aggarwal and Nicholson, 2002).

By collecting tissues from mice at different disease stages, we studied populations of mutant SOD1 transgenic mice synchronized for their disease stages. These studies revealed several surprises. First, at the RD stage when the disease began (60 to 90 days before paralysis), the loss of motor neurons was minor (less than 10%). The largest loss of motor neuron occurred at the paralysis stage (Fig. 1). This pattern correlated with the changes in astrogliosis, which also sharply rose at the paralysis stage (Levine *et al.*, 1999; Fig. 1). This suggested that early therapeutic intervention after onset of ALS may rescue the majority of motor neurons. Second, deliberate searching for prominent neurofilament abnormalities revealed few sites of neurofilament accumulation. These minor changes occurred mostly in late SD and paralysis stages. In contrast, widespread vacuoles in the spinal cord were easily observed without deliberate searches. By quantitative measurements, the number of these vacuoles peaked at the RD stage. As the disease further progresses towards the paralysis stage, the number of vacuoles declined (Kong and Xu, 1998; Fig. 1).

The peaking of vacuoles in the ventral horn was particularly interesting because it represented a dominant early pathological event. The follow-up detailed microscopic observations confirmed that these vacuoles were derived from vacuolated mitochondria (Kong and Xu, 1998). Tracing back before the onset of the disease and massive mitochondrial vacuolation, we found a large number of abnormal mitochondria associated with neuronal processes, predominantly in dendrites (Kong and Xu, 1998). This result indicated that neuronal mitochondrial damage began early, prior to the clinical onset of ALS. Indeed, functional measurement of mitochondrial complex I activity was detected decline at age 60 days, the earliest age that we measured (Jung *et al.*, 2002; Fig. 1).

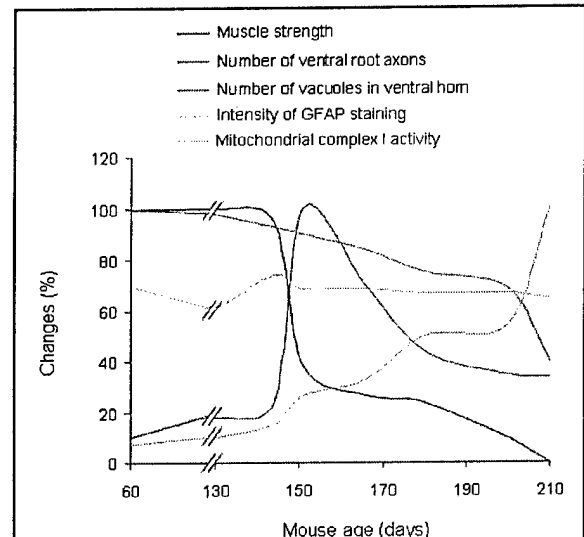


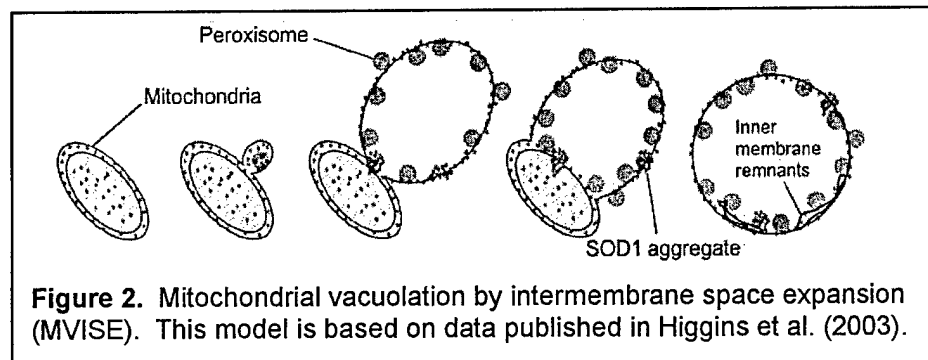
Figure 1. Sequence of pathological events leading to motor neuron death in mutant SOD1G93A mice. This plot is based on data in Kong and Xu (1998), Levine *et al.* (1999) and Jung *et al.* (2003).



Mitochondrial vacuolation by intermembrane space expansion (MVICE)

How do mitochondria become vacuolated in the SOD1G93A mutant mice? Our electron microscopic observations suggested a pattern of progression in mitochondrial vacuolation (Higgins *et al.*, 2003; Fig. 2). Initially mitochondria are swollen and their cristae are disorganized. But they maintain the general structure of a mitochondrion. Then the outer membrane folds at a focal point, forming a small protrusion on the mitochondrial surface and creating a small space between the outer and inner membranes. This might be caused by damage to the attachment structure between the inner and outer mitochondrial membranes. Following the formation of this small protrusion is a further detachment between the inner and outer membranes and expansion of the intermembrane space. As the space becomes increasingly large, the inner membrane components disintegrate, forming the inner membrane remnants inside the mitochondrial vacuole (Higgins *et al.*, 2003; Fig. 2).

This model for mitochondrial vacuolation was plausible because several other studies have shown that both wild type and mutant SOD1 exist in mitochondrial intermembrane space (Okado-Matsumoto and Fridovich, 2001; Sturtz *et al.*, 2001; Higgins *et al.*, 2002; Mattiazzi *et al.*, 2002) and expansion of intermembrane space had been suggested (Bendotti *et al.*, 2001; Jaarsma *et al.*, 2001).



To test this model, we localized markers for various mitochondrial components in the vacuoles. We took the advantage of our early finding that mutant SOD1 was present at the boundary of vacuoles {Levine, 1999 #461} and marked vacuoles using anti-SOD1 antibodies. By double immunofluorescent staining, we demonstrated that the inner mitochondrial membrane marker cytochrome c oxidase was located with the inner membrane remnants inside the vacuole. The outer mitochondrial membrane markers, transporter of outer membrane TOM20 and TOM40, were located on the outer vacuolar membrane. Cytochrome c, an intermembrane space marker, colocalized with SOD1 in mitochondria at the beginning stage of the vacuolation, but disappeared when the vacuoles enlarged. The disappearance of cytochrome c in large vacuoles could be either due to a dilution of cytochrome c as the intermembrane space expands, or due to leakage out of mitochondrial intermembrane space. Our observation that the outer membranes of the large vacuoles were often porous supported the latter possibility (Higgins *et al.*, 2003).

As we further looked for other organelles that might participate in mitochondrial vacuolation, we were surprised to find abundant peroxisomes inside the vacuoles and that lysosomes were not associated with the vacuoles (Higgins *et al.*, 2003). The features of this mitochondrial vacuolation, including the expansion of the intermembrane space (instead of mitochondrial matrix) and the presence of peroxisomes (instead of lysosomes), suggest that the mutant SOD1-induced mitochondrial vacuolation is neither the classical mitochondrial permeability transition (which involves expansion of the mitochondrial matrix) nor autophagic vacuolation (which involves lysosomes), but rather, a vacuolation by intermembrane space expansion or MVICE (Fig. 2).



The role of mitochondrial degeneration in motor neuron degeneration pathway

Experiments in cultured cells and in mice from other investigators support our *in vivo* observations on mutant SOD1-induced mitochondrial degeneration. Expression of mutant SOD1 in cultured neuroblastoma cells caused a decrease in mitochondrial membrane potential (Carri *et al.*, 1997). Similarly, expression of mutant SOD1 in motor neuron-like cell line NSC34 cells damaged mitochondria, caused mitochondrial dysfunction and cell death (Liu *et al.*, 2002; Menzies *et al.*, 2002b). Targeting mutant SOD1 to mitochondria in cultured neuroblastoma cells produced heightened toxicity (Takeuchi *et al.*, 2002). *In vivo*, transgenic mice expressing mutant SOD1 showed a loss in mitochondrial mass (Wiedemann *et al.*, 2002). Mitochondrial toxin MTPT or a decrease in mitochondrial SOD2 activity significantly exacerbated the clinical progression of ALS in mutant SOD1 transgenic mice (Andreassen *et al.*, 2000; Andreassen *et al.*, 2001).

Is mitochondrial degeneration relevant for human ALS? Although early studies on human autopsy focused on neurofilament accumulation in proximal axons because of their conspicuous presence, careful examination of the published EM micrographs reveals numerous vacuolated mitochondria among swirls of disorganized neurofilaments in both sporadic and familial cases (Hirano *et al.*, 1984a; Hirano *et al.*, 1984b). Other studies have shown vacuolated mitochondria in upper and lower motor neurons (Sasaki *et al.*, 1990; Sasaki and Iwata, 1999) as well as hepatocytes (Masui *et al.*, 1985) in human ALS cases. These observations suggest that mitochondrial degeneration play a role in human ALS.

Some SOD1 mutants cause motor neuron degeneration without mitochondrial vacuolation (Ripps *et al.*, 1995; Bruijn *et al.*, 1998; Wang *et al.*, 2002). However, whether these mutants cause functional impairment is unclear and remains a future challenge for our investigation. To further understand the role of mitochondrial damage in the motor neuron degeneration pathway, two questions need to be answered: how do SOD1 mutants damage mitochondria and what is the consequence of this damage? Mutant SOD1 may directly damage mitochondria. Both wild type and mutant SOD1 are found in mitochondria (Higgins *et al.*, 2002; Mattiazzi *et al.*, 2002), probably in the intermembrane space (Okado-Matsumoto and Fridovich, 2001; Sturtz *et al.*, 2001). Mutant SOD1 aggregates in mitochondria and the aggregates are associated with mitochondria membranes (Higgins *et al.*, 2003). Therefore, the aggregates could cause direct damage to mitochondrial membranes. Evidence for protein aggregates damaging biomembranes has emerged from studies on Parkinson's disease, where mutant α -synucleins forms aggregates and annular rings on membranes (Lashuel *et al.*, 2002), and causing permeabilization of membranes (Volles *et al.*, 2001). Mutant SOD1 could damage mitochondria by similar mechanisms.

Mutant SOD1 could also damage mitochondria indirectly. Mutant SOD1 interacts with cellular chaperones (Shinder *et al.*, 2001; Okado-Matsumoto and Fridovich, 2002) and inhibits chaperone activity in motor neurons (Bruening *et al.*, 1999; Batulan *et al.*, 2003). Mitochondrial protein import depends on cytoplasmic as well as mitochondrial chaperone activities (Neupert and Brunner, 2002; Young *et al.*, 2003). The reduced chaperone activity may impair mitochondrial function by interfering with protein import into mitochondria.

What is the consequence of mitochondrial damage? Mitochondrial dysfunction can lead to energy deficiency and ionic imbalance (Beal, 1992), elevated reactive oxidative stress and oxidative damage (Andreassen *et al.*, 2000), and increased sensitivity of neurons to excitotoxicity (Ikonomidou *et al.*, 1996; Bittigau and Ikonomidou, 1997; Kruman *et al.*, 1999; Kaal *et al.*, 2000). These effects could lead to structural damage to mitochondria, resulting in MIVSE. The loss of structural integrity could trigger cell death programs by releasing pro-apoptotic proteins that reside in the mitochondrial intermembrane space, such as cytochrome c, AIF, SMAC/DIABLO, endo G and Htra/Omi (Green and Evan, 2002). Because the majority of vacuoles develop in distal small dendrites (Levine *et al.*, 1999), the release of these proapoptotic molecules may not cause typical



apoptotic changes in motor neuron cell bodies, such as chromatin condensation and cytoplasmic blebbing. Indeed, the typical changes are not observed by EM (Bendotti *et al.*, 2001; Guegan and Przedborski, 2003), but widespread caspase activation is detected in spinal cords (Pasinelli *et al.*, 2000; Guegan *et al.*, 2001; Guegan and Przedborski, 2003), suggesting the occurrence of a neuritic death program (Mattson and Duan, 1999).

In summary, there is strong evidence that mitochondrial degeneration plays important roles in mutant SOD1-induced motor neuron degeneration pathway. Further challenges in defining this role will be to determine whether SOD1 mutants that do not induce mitochondrial vacuolation impair mitochondrial function and the mechanism whereby all mutant SOD1 damages mitochondria. Because of the complexity of cellular environment, further breakthrough in mechanistic understanding of how mutant SOD1 damages mitochondria will most likely emerge from defined in vitro experimental systems.

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POSTER

ABSTRACTS



1

ON THE NATURE OF CYCLOSPORIN NEUROPROTECTIVE EFFECT: THE MITOCHONDRIA CONNECTION

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It is well established that cyclosporin A (CsA) is neuroprotective and recent data suggest that this effect does not depend on immunosuppression (calcineurin inhibition). Here we investigated the mechanism of action of CsA and its nonimmunosuppressive analogues SDZ 211811 and CsH in further detail. Immunohistochemistry and Western blot analysis were used to investigate the possible modulation of Bcl-2 and Bcl-x_L protein levels and distribution in cultured hippocampal neurons as well as differentiated SH-SY5Y neuroblastoma cells. Pre-treatment with CsA enhanced the expression level and altered the expression pattern of Bcl-2 and Bcl-x_L proteins compared to vehicle treated control cultures. These effects were mimicked by the non-immunosuppressive CsA analogue SDZ 211811 as well as CsH (a derivative that does not bind to cyclophilins) indicating that drug binding to known cyclophilins was not involved. Enumeration of mitochondria in neurites showed that CsA/CsH treatment increased the number of mitochondria in the neurites. This altered distribution of mitochondria was also independent of drug binding to cyclophilins. These data suggest that effects of cyclosporin on Bcl protein expression as well as mitochondrial distribution may contribute to their neuroprotective effect and that these effects are not mediated by direct inhibition of calcineurin or cyclophilin D. This work was supported by DFG grant Ja865/1-1 to R.J.

2

CALCIUM-INDUCED DAMAGE TO NEURON AND ASTROCYTE MITOCHONDRIA AND PROTECTION BY PERMEABILITY TRANSITION INHIBITORS

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This study tested the hypothesis that the sensitivity of mitochondria to the Ca²⁺-induced loss of mitochondrial membrane potential ($\Delta\psi_m$) and the sensitivity of the loss of $\Delta\psi_m$ to mitochondrial permeability transition (MPT) inhibitors are different for neurons and astrocytes. Experiments with digitonin-permeabilized primary cultures of cerebellar granule neurons and cortical astrocytes measured the capacity of mitochondria to accumulate and retain exogenous Ca²⁺. The Ca²⁺ uptake capacity of astrocytes was four-fold greater per cell than that of cerebellar granule neurons. As astrocytes contain twice the mitochondrial DNA than neurons, the Ca uptake capacity per mitochondrion for astrocytes was approximately twice that of neurons. Moreover, the uptake capacity of astrocytes was 100% higher in the presence of cyclosporin A (CsA) while the mitochondrial Ca²⁺ uptake of neurons was unaffected. FK506, another immunophilin drug that fails to inhibit MPT in isolated mitochondria, had no effect on Ca²⁺ uptake by either digitonin-permeabilized astrocytes or neurons. To study the effect of Ca²⁺ on $\Delta\psi_m$ in intact cells, cultures of rat cortical neurons and astrocytes were exposed to the Ca²⁺ ionophore 4-Br-A23187 (5 μ M) and $\Delta\psi_m$ was monitored with the fluorescent probe TMRM. 4-Br-A23187 caused a decline in $\Delta\psi_m$ in both cell types that was inhibited by CsA and FK506 in astrocytes but not neurons. Paradoxically, other MPT inhibitors, 2-APB and bongrekic acid failed to protect against Ca²⁺-induced decline in $\Delta\psi_m$ in astrocytes. 4-Br-A23187-mediated cytosolic Ca²⁺ rise [Ca²⁺]_i (measured by Fura FF) was significantly higher in neurons than astrocytes. Preincubation with CsA and FK506 significantly reduced the increase in [Ca²⁺]_i in both cell types. We conclude that the protective effect of CsA and FK506 against Ca²⁺ ionophore-induced mitochondrial membrane depolarization in intact astrocytes is not due to MPT inhibition but might be due to decrease in [Ca²⁺]_i rise. (Supported by NIH grant NS34152)



3

NON-IMMUNOSUPPRESSIVE CYCLOSPORIN ANALOGS INHIBIT MITOCHONDRIAL PERMEABILITY TRANSITION AND NEURONAL DEATH IN HIPPOCAMPAL SLICE CULTURES

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Cyclosporin A (CsA) has been shown to be neuroprotective in animal models of ischemia, traumatic brain injury, hypoglycemic coma as well as prolonging survival of transgenic ALS mice. Inhibition of the mitochondrial permeability transition (mPT) has emerged as a possible mechanism for the powerful *in vivo* neuroprotection displayed by CsA. In the present ongoing study, we have evaluated new non-immunosuppressive cyclosporin analogs (NIM-811, Novartis and UNIL025, Debiopharm) for their ability to inhibit mPT in rodent brain-derived mitochondria as well as their prevention of cell death in an *in vitro* model of ischemic brain damage (described in Rytter A. *et al.* JCBF 23:23-33, 2003). Both NIM-811 and UNIL025 were found to be powerful inhibitors of calcium-induced mitochondrial swelling under energized and de-energized conditions and the effects were comparable to those of native CsA. In addition, both CsA and the first non-immunosuppressive cyclosporin analog tested ameliorated selective CA1 cell death in organotypic mouse hippocampal slices exposed to 15 min of oxygen and glucose deprivation. Stringent evaluation of the efficacy displayed by CsA and its non-immunosuppressive analogs can aid in discriminating between (i) inhibition of the calcium-activated phosphatase calcineurin and (ii) counteracting the mitochondrial permeability transition as the primary neuroprotective mechanism displayed by these compounds.

4

MITOCHONDRIAL PERMEABILITY TRANSITION PORES AS A TARGET FOR NEUROTOXIC AND NEUROPROTECTIVE AGENTS.

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It is generally recognized now that mitochondrial permeability transition (MPT) pores play a key role in the maintenance of Ca^{2+} homeostasis in the cell and in apoptosis and necrosis processes. The main goal of the present work was to study the effect of different model neurotoxins, used for simulating of such neurodegenerative disorders as Alzheimer's disease (AD) and parkinsonism as well as series of known neuroprotective agents on MPT functioning. First, it was demonstrated that parkinsonogenic neurotoxin methylphenyl-pyridinium (MPP), as well as AD-simulating neurotoxins: ethylcholine aziridinium (AF64A) and synthetic fragment of β -amyloid peptide – $\text{AP}\beta$ (25-35) all induced the cyclosporin-A specific opening of MPT pores. Secondly, we have shown that such anti-Alzheimer's drugs as Tacrine, Memantine, or antihistaminic agent Dimebon, proposed recently for AD treatment (US Patent 6,187,785; EP 0 876 818 B1) all inhibited the calcium-induced MPT. The similar effect was revealed also for some natural and endogenous compounds, possessing neuroprotective properties. In particular, extract of *Ginkgo biloba* (EGb 761) and endogenous melatonin precursor N-acetylserotonine (in contrast to melatonin itself) both inhibited the t-butylhydroperoxide-induced MPT. On the ground of these results it was suggested that the induction of MPT can be the common link in the mechanisms of different neurodegenerative disorders and, from the other hand, MPT pores might be discussed as promising target for the new generation of neuroprotective agents (mitoprotectors).



5

CALPAIN I INDUCES MITOCHONDRIAL OUTER MEMBRANE PERMEABILITY IN THE ABSENCE OF PERMEABILITY TRANSITION

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Calpain activation occurs during glutamate excitotoxicity and has been implicated in both acute and chronic neurodegenerative disorders (e.g. cerebral ischemia/reperfusion brain injury and Huntington's disease). We tested the hypothesis that calpain I (activated by micromolar levels of calcium) can directly induce mitochondrial outer membrane permeability and release of apoptogenic proteins via the cleavage and activation of one or more Bcl-2 family proteins. Calpain treatment of isolated mouse liver mitochondria induced dose-dependent Bak cleavage and cytochrome *c* release in the absence of mitochondria permeability transition and in a calpeptin-inhibitable fashion. Nevertheless, mitochondria isolated from the livers of Bak-deficient mice were fully competent in releasing cytochrome *c* in response to calpain. Adult mouse brain mitochondria, which contain lower levels of Bak with respect to liver mitochondria and virtually undetectable levels of Bax, were refractory to Bak cleavage and cytochrome *c* release. The addition of recombinant full-length monomeric Bax to isolated mouse liver mitochondria augmented cytochrome *c* release induced by calpain and was associated with Bax processing to an 18-kDa fragment. In contrast to calpain, caspases-2 and -3 were unable to induce cytochrome *c* release from mouse liver mitochondria in the absence of inner membrane permeability transition. Experiments are in progress to determine whether liver mitochondria and immature brain mitochondria isolated from Bax-deficient mice are resistant to calpain I-induced cytochrome *c* release.

6

RELEASE OF CYTOCHROME C FROM BRAIN MITOCHONDRIA INDUCED BY CASPASE 8-CLEAVED BID AND BAX.

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Cytochrome *c* (Cyt *c*) release from isolated, non-synaptosomal, Percoll gradient-purified brain mitochondria and concomitant changes in mitochondrial functioning induced by recombinant, caspase 8-cleaved (truncated) BID (tBID) and monomeric full-length BAX or C-terminal deletion mutant of BAX (BAXΔC) have been characterized. A capture ELISA was used to measure Cyt *c* release. The protein-induced Cyt *c* release was compared with the maximal alamethicin-induced release. tBID alone caused partial Cyt *c* release representing 20% of alamethicin-induced release. Neither monomeric BAXΔC nor full-length monomeric BAX induced Cyt *c* release. A combination of tBID with full-length BAX was more efficient than a combination of tBID with BAXΔC, releasing 90 and 45% of Cyt *c*, respectively. The release of Cyt *c* induced by tBID and full-length BAX was accompanied by mild depolarization of mitochondria without overt changes of mitochondrial morphology as judged by transmission electron microscopy. In contrast to Cyt *c*, another mitochondrial apoptogenic protein Smac/DIABLO was not released after treatment of mitochondria with a combination of tBID and full-length BAX or with alamethicin. A mixture of tBID and full-length BAX activated ROS generation and release of free fatty acids (FFA) from brain mitochondria monitored with Amplex Red and with acrylodated intestinal fatty acid binding protein (Molecular Probes), respectively. Both activation of ROS generation and release of FFA appeared to precede the release of Cyt *c*. The amphiphilic cation propranolol inhibited all three processes. Possible interrelations between Cyt *c* release, activation of ROS generation, and activation of FFA production are discussed.

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TAT-MEDIATED INTRACELLULAR DELIVERY OF THE LOOP DELETION BCL-2 PROTEIN PROTECTS NEURONS AGAINST CELL DEATH.

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Protein transduction mediated by permeability transduction domain (PTD) containing peptides such as the HIV-1 Tat peptide has recently emerged as a promising approach for neuroprotection. The objective of this project was to generate and evaluate the neuroprotective potential of Tat-fusion proteins with anti-apoptotic Bcl-2 family proteins. We report here the generation and characterization of a Tat-Bcl-2 construct with the loop domain deleted (Tat-Bcl-2 Δ loop). Deletion of this domain that contains several phosphorylation sites and the caspase-3 cleavage site was previously reported to enhance the anti-apoptotic activity of Bcl-2 in non-neuronal cells. This construct was tested here for its ability to enhance neuronal survival. The Tat-Bcl-2 Δ loop construct was generated by ligation of the Tat sequence in frame with the human Bcl-2Dloop cDNA. The protein was over-expressed in the C41(DE3) and C43(DE3) *E. Coli*, and then purified. The results obtained indicate that recombinant Tat-Bcl-2 Δ loop protein is functional and that protein transduction can be achieved in both neuronal cell lines and primary hippocampal neurons. The purified Tat-Bcl-2 Δ loop is able to associate with mitochondria as determined in an *in vitro* assay with mitochondria isolated from GT1-7 neural cell line and isolated rat brain mitochondria. Experiments with GT1-7, PC12 and SH-SY5Y cells showed that Tat-Bcl-2 Δ loop protein transduces cultured cells in a dose-dependent manner, when used at nM concentrations, and protects both PC12 and GT1-7 cells from staurosporine-induced death. The effect of Tat-Bcl-2 Δ loop on B27 withdrawal-induced death was examined in primary hippocampal neurons. Pre-treatment with Tat-Bcl-2 Δ loop (100nM) was able to enhance by 60% the survival of hippocampal neurons cultured for 44 hours in the absence of B27 supplement. These results support the idea that enhanced neuroprotection might be achieved by using modified Bcl-2 protein constructs, such as the loop deletion Bcl-2 protein tested here. (Supported by NIH grant NS45038)

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POLY(ADP-RIBOSE) POLYMER SERVES AS A DEATH SIGNAL BY TRIGGERING APOPTOSIS-INDUCING FACTOR RELEASE

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Following NMDA insult, translocation of AIF from the mitochondria to the nucleus is PARP-1-dependent but caspase-independent. To determine how nuclear PARP-1 activation leads to AIF translocation, we assessed the relationships between intracellular energy depletion and translocation of AIF in cortical neurons. Under the condition of 500 μ M NMDA stimulation (5 min), we observe robust PARP activation, ATP and NAD⁺ decrements, and progressive AIF translocation. When the neurons were treated with oligomycin (30 μ M), ATP and NAD⁺ levels were almost completely lost after 8 h, but there is no significant release of AIF nor is there PARP-1 activation. To examine whether PAR polymer serves as a death signal, we delivered purified PAR polymer into cultured neurons with the BioPorter reagent. Immunochemical staining indicates that PAR polymer can be effectively delivered into neurons and induces AIF release and neuronal death. In a cell free system, incubation of PAR polymer with the isolated neuronal mitochondria elicits mitochondrial membrane depolarization and AIF release. Pretreatment of the purified PAR with either PARG or phosphodiesterase I, which degrade PAR polymer into monomers, can completely abolish AIF release and the death-inducing effect in these assays. Additionally, immunochemical staining, subcellular fractionation and Western blotting showed that PAR can be detected in the cytoplasm after 15 min of NMDA treatment. Delivery of a neutralizing anti-PAR antibody or overexpression of a PARG-adenoviral construct in cultured cortical neurons can reduce NMDA induced excitotoxicity. These results taken together suggest that energy depletion is a biomarker for PARP-1 activation but is not sufficient to elicit AIF-dependent cell death and instead PAR polymer can serve as a death signal by triggering AIF release and neuronal death. Support Contributed By: AHA and NIH



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ASSOCIATION OF ^{31}P NMR MEASUREMENTS OF H_2O_2 -INDUCED ENERGY FAILURE IN NEONATAL RAT CEREBROCORTICAL SLICES WITH PAR-POLYMER FORMATION AND RESCUE BY PYRUVATE WITH GLUCOSE

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Published neuron and neuron-astrocyte cell culture studies report excessive PARP activation after exposure to the DNA alkylating agent MNNG or to H_2O_2 . Also found were NAD depletion, glycolytic arrest, and cell death that could be prevented or reversed by PARP inhibitors or TCA cycle metabolites, including pyruvate. To determine if similar PARP-associated energy failures occur in neonatal rat cerebrocortical slices, 14.1 Tesla $^{31}\text{P}/^1\text{H}$ NMR spectroscopy studies of perchloric acid (PCA) extracts, used to estimate the time course of ATP, ADP, PCr and NAD levels, were compared with immunoblots, immunostains, and other histological and cellular measures. Slices 350 μm thick, obtained from P7 Sprague-Dawley rats, underwent 3 h post-decapitation metabolic recovery during superfusion with 37°C oxygenated artificial cerebrospinal fluid (ACSF). Thereafter normal oxy-ACSF with 10 mM glucose was replaced by oxy-ACSF having H_2O_2 (2 mM or 4 mM) without glucose. Exposures to H_2O_2 , which has PARP activation among its effects, lasted 1 hour. Thereafter, 4 hrs superfusion occurred with ACSF having pyruvate and glucose. In some experiments the PARP inhibitor 3-aminobenzamide (3AB, 3 mM or 5 mM) was also administered, this starting 1 hour prior to H_2O_2 administration and stopping when H_2O_2 exposure ceased. Data were compared from slices taken at four time points: 1) $t=0$ h, just before H_2O_2 exposure; 2) $t=20$ min, during H_2O_2 exposure; 3) $t=60$ min, the end of H_2O_2 exposure; and 4) $t=5$ hr, after 4 hrs of post-treatment. NMR spectra showed substantial decreases in all high energy phosphates at $t=1$ h, these being largely preventable with 3 mM 3AB. Spectral resolution at 14.1 T could resolve ADP from ATP as well as the resonance for NAD(H)/NADP(H) from resonances for UDP-sugars. Western blots and immunohistology showed substantial PAR-polymer formation after H_2O_2 insults, these also being effectively prevented by 3AB, and NAD losses measured in enzymatic assays. Energy recovery in ^{31}P NMR spectra occurred when various pyruvate-glucose combinations were used during the 4 hr recovery period. Our brain slice model demonstrates associations between PARP, energy failure, and energy recovery.

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MITOCHONDRIAL IMAGING IN NIGROSTRIATAL ORGANOTYPIC COCULTURE

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Parkinson's disease (PD) is a slow progressive disorder. However, many of the experimental studies in the past have relied on some form of acute toxic injury to model this condition. The sequence of events culminating in neurodegeneration in response to an acute injury may not be the same as that of a chronic injury. Accurate identification of the sequence of events of neurodegeneration, especially the early events, may provide vital insights to the pathogenesis and treatment of PD. The "roller-tube" nigrostriatal organotypic culture can serve as a reliable long-term *in vitro* model that is ideally suited for studying the physiology of both the terminal and cell body of dopamine neurons. Explants from the Substantia nigra and striatum are cocultured. In culture the nigral dopaminergic neurons form projections to appropriate target neurons in the striatal explant. The thinness of the mature cultures makes them well suited for imaging studies. Because these cultures can be maintained for ten weeks or more, they provide an ideal preparation for chronic treatment studies. We have undertaken mitochondrial imaging and immunohistochemical studies to determine whether physiological dysfunction and degeneration in early PD affects all parts of the neuron equally or simultaneously. We have shown that the number of dopaminergic (tyrosine hydroxylase+) neurons in culture remain stable for at least 50 days. We have measured mitochondrial membrane potential (MMP) using TMRM in cultures ranging from 40 to 80 days *in vitro*. In these imaging studies, we have demonstrated dose-dependent effects (1 nM to 30 μM) of FCCP and rotenone in both neuronal somata and projections. Rotenone concentrations below 30 nM have no acute effect on MMP. This system allows us to compare the effects on the soma to the effect at the nerve terminals and assess effects of treatments such as rotenone. *Supported by VA REAP Award to the Baltimore VAMC (S.J., C.-M.T.) and NIH NS34152*



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CCCP REDUCES EVOKED NEUROTRANSMITTER RELEASE BUT ENHANCES SPONTANEOUS NEUROTRANSMITTER RELEASE

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The consequences of synaptic mitochondrial dysfunction are being examined in the hippocampal CA1 region of brain slices from young (18 – 25 days) rats. Mitochondrial dysfunction is induced by CCCP with or without oligomycin. Because mitochondria play a crucial role in ATP production, Ca^{2+} regulation, and free radical production, damages in mitochondria can lead to many problems including raised intracellular Ca^{2+} . In the presence of a mitochondrial uncoupler, CCCP, the postsynaptic cells preserved their intrinsic excitability, being able to generate action potentials, yet synaptic transmission was altered. Intracellular recordings in CA1 layer showed a marked decrease in EPSP-IPSPs evoked by stratum radiatum stimulation. The IPSP was first affected, followed by reduction of the EPSP. 10 μM of CCCP with 10 $\mu\text{g/ml}$ oligomycin completely abolished the EPSP-IPSP within 4 minutes of drug application. When a lower dose of CCCP (2 μM without oligomycin) was applied, the EPSP-IPSP decrease was much slower (approximately 20min for complete blockade). CCCP also altered spontaneous neurotransmitter release. With a low dose of CCCP (2 μM), the change was difficult to measure, but with a higher dose (10 μM), the frequency of spontaneous EPSPs was increased by 36%. The increase of spontaneous neurotransmitter did not happen initially, and sometimes even decreased frequency was observed. Increased frequency occurred after the evoked EPSP-IPSPs were significantly reduced. We hypothesize that these changes in synaptic transmission are due to raised intracellular Ca^{2+} from mitochondrial dysfunction, but the exact mechanism of decreased evoked and increased spontaneous neurotransmitter release is under further investigation. Supported by CIHR and the Alzheimer's Association.

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STUDIES OF SINGLE ISOLATED MITOCHONDRIA REVEAL DIFFERENT PROPERTIES OF Ca^{2+} -INDUCED DEPOLARIZATION IN BRAIN AND LIVER MITOCHONDRIA

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We used fluorescence microscopy to examine the effects of Ca^{2+} on membrane potential ($\Delta\Psi\text{m}$) in individual mitochondria adhered to glass coverslips. As reported previously, single brain mitochondria in K^{+} -based buffer exhibit spontaneous, large-amplitude fluctuations of $\Delta\Psi\text{m}$, as measure by the potentiometric fluorophore rhodamine 123 (Vergun et al, Biophys.J., 2003). Here we show these fluctuations are inhibited by Ca^{2+} chelation, and resume upon the addition of small amounts of Ca^{2+} . Excessive Ca^{2+} (35 μM) initiated a rapid and complete depolarization of all mitochondria. ATP or ADP abolished spontaneous activity, and also delayed high Ca^{2+} -induced depolarization. The effects of ATP and ADP were abolished by inhibition of nucleotide transport with carboxylatractyloside. None of the phenomena were sensitive to cyclosporine A (CsA).

In contrast to brain mitochondria, $\Delta\Psi\text{m}$ of liver mitochondria showed no spontaneous oscillations, and addition or chelation of Ca^{2+} did not initiate oscillatory activity. As with brain mitochondria, high added Ca^{2+} (35 μM) caused complete and rapid depolarization. Furthermore, Ca^{2+} -induced depolarization was similarly delayed by nucleotides in a carboxylatractyloside-sensitive manner. In contrast to brain mitochondria, CsA in liver mitochondria significantly delayed the onset of Ca^{2+} -induced depolarization, and greatly altered the kinetic of depolarization between individual mitochondria. These results illustrate that $\Delta\Psi\text{m}$ is regulated differently in brain and liver mitochondria, and that $\Delta\Psi\text{m}$ oscillations may be a characteristic of brain but not liver tissue. Supported by NIH grant NS41299 (IJR).



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MITOCHONDRIAL MEMBRANE POTENTIAL ALTERED BY OXYGEN-GLUCOSE DEPRIVATION AND SUBSEQUENT APOPTOSIS IN RAT PRIMARY HIPPOCAMPAL CULTURE.

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Introduction: An observation system of mitochondrial membrane potential (MMP) in an anaerobic chamber was developed and examined the influence of oxygen-glucose deprivation (OGD) on MMP and the subsequent apoptosis.

Method and Material: Primary hippocampal cell culture in a medium without glucose was placed on an tightly-sealed chamber mounted on the microscope. JC-1 was added for evaluation of $\Delta \psi_m$. The chamber was filled with 95% N₂ and 5% CO₂ for 30 or 90 min following reoxygenation. The viability and death mode were evaluated at 3 hrs and 24 hrs of reoxygenation.

Results: MMP turned depolarized after approximately 20 min of OGD, although the change varied between neurons. Then, MMP hyperpolarized at 30 min reoxygenation following 30OGD, while MMP remained depolarized at 30 min reoxygenation following 90OGD. Most neurons were viable (76%) at 3 hrs following 30OGD, while most neuron was dead (72%) at 3 hrs following 90OGD. Most neurons (66%) were TUNEL positive at 24 hours following 30OGD. Only a few neurons (12%) were TUNEL positive following 90OGD.

Discussions: We demonstrated that long OGD results in depolarization, while shorter OGD induces hyperpolarization. Neurons are still viable during hyperpolarization, but this hyperpolarization seems to link the subsequent manifestation of apoptosis.

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HYPOXIA INDUCES CYTOCHROME C RELEASE FROM ONLY A SUBPOPULATION OF MITOCHONDRIA IN HIPPOCAMPAL NEURONS

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Recent evidence suggests that following hypoxia neuronal mitochondria release cytochrome c (cyt c), an important step in delayed cell death, but the mechanism of release and the mode of death under these conditions are controversial. We previously showed that excitotoxic NMDA stimulation of hippocampal neurons led to heterogeneous mitochondrial Ca overload and swelling, resulting in release of cyt c from an injured subpopulation of mitochondria while other mitochondria continued to function normally. These conditions are favorable for apoptosis. During staurosporine-induced apoptosis cyt c was also incompletely released from mitochondria, as indicated by the observation that even at late stages of apoptosis neurons retained cyt c in their reduced complement of remaining mitochondria. Using EDX microanalysis, fluorescence microscopy and EM immunocytochemistry, we found that cyanide (10 mM, 30 min) induced intracellular redistribution of Na and K and rapid Ca accumulation in both cytoplasm and mitochondria of neurons, accompanied by depolarization of the mitochondrial membrane potential (MMP). Although cyanide-induced Ca accumulation was not sensitive to the NMDA receptor antagonist MK-801, these changes were similar to those observed during NMDA excitotoxicity, as was the distribution of cyt c. This protein is normally localized to mitochondria, but 30 min after stimulus removal it became diffusely distributed in the cytoplasm but also retained in undamaged mitochondria. The majority of neurons did not die early by necrosis, but instead recovered normal volume, ion composition and MMP after cyanide removal; many of these later developed signs of apoptosis similar to those seen in excitotoxicity. In particular, degenerating neurons retained cyt c in remaining mitochondria, implying that only a subpopulation of mitochondria was sufficiently injured by Ca overload to trigger cyt c release. The results indicate that delayed neuronal death induced by hypoxia, excitotoxic stimulation, or staurosporine similarly involves cyt c release from a subpopulation of injured mitochondria.



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MITOCHONDRIAL PERMEABILITY TRANSITION IN NEURONAL DAMAGE PROMOTED BY Ca^{2+} AND RESPIRATORY CHAIN COMPLEX II INHIBITION

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Changes in mitochondrial integrity, reactive oxygen species release and Ca^{2+} handling are proposed to be involved in the pathogenesis of many neurological disorders including methylmalonic acidemia and Huntington's disease, which exhibit partial mitochondrial respiratory inhibition. In this report, we studied the mechanisms by which the respiratory chain complex II inhibitors malonate, methylmalonate and 3-nitropropionate affect rat brain mitochondrial function and neuronal survival. All three compounds, at concentrations which inhibit respiration by 50%, induced mitochondrial inner membrane permeabilization when in the presence of micromolar Ca^{2+} concentrations. ADP, cyclosporin A and catalase prevented or delayed this effect, indicating it is mediated by reactive oxygen species and mitochondrial permeability transition (PT). PT induced by malonate, methylmalonate and 3-nitropropionate was also present in mitochondria isolated from liver and kidney, but required more significant respiratory inhibition. In brain, PT promoted by complex II inhibitors was stimulated by increasing Ca^{2+} cycling and absent when mitochondria were pre-loaded with Ca^{2+} or when Ca^{2+} uptake was prevented. In addition to isolated mitochondria, we determined the effect of methylmalonate on cultured PC12 cells and freshly prepared rat brain slices. Methylmalonate promoted cell death in striatal slices and PC12 cells, in a manner attenuated by cyclosporin A and bongkrekate. We propose that under conditions in which mitochondrial complex II is partially inhibited in the central nervous system, neuronal cell death involves the induction of PT.

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THE ROLE OF OXIDATIVE STRESS IN THE IMPAIRMENT OF ISOLATED BRAIN MITOCHONDRIA BY Ca^{2+} AND HYPOXIA/REOXYGENATION

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Animal models of stroke have demonstrated that brain mitochondria are deeply involved in neurodegenerative processes within the infarcted area. The change of hypoxia and reoxygenation in combination with elevated cytosolic Ca^{2+} concentrations is supposed to cause mitochondrial damage, which is mediated by oxidative stress. However, clinical interventions using antioxidative treatments were only partially successful. Therefore this study aimed to investigate whether mitochondrially derived reactive oxygen species (ROS) are involved in damaging of the organelle. Therefore, we subjected isolated rat brain mitochondria in the presence of micromolar Ca^{2+} to hypoxia followed by reoxygenation and determined the following parameters: respiration, membrane permeability, glutathione oxidation, extramitochondrial H_2O_2 level and morphology. The treatment described above performed in the presence of extramitochondrial AMP led to functional breakdown, cyclosporin A-insensitive membrane permeabilisation, glutathione oxidation, and disintegration of the mitochondria. In contrast, ADP completely protected isolated brain mitochondria from membrane permeabilisation and functional breakdown. The permanent presence of a ROS detoxifier (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPOL), which is able to permeate the mitochondrial membrane system did not prevent functional and morphological injury. Moreover, increase in extramitochondrial H_2O_2 concentration was lower after hypoxia/reoxygenation, which was applied simultaneously with micromolar extramitochondrial Ca^{2+} , in comparison to normoxic controls. From our data we conclude that mitochondrially induced oxidative stress, determined as glutathione oxidation, is the consequence but not the cause of mitochondrial injury by hypoxia/reoxygenation and Ca^{2+} . Thus, a key step in the process of mitochondrial damage is membrane permeabilisation, which can be prevented by ADP.



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ROS-INDUCED CARDIOLIPIN PEROXIDATION BY COMPLEX I INHIBITORS FACILITATES THE RELEASE OF CYTOCHROME C BY PRO-APOPTOTIC MOLECULES: RELEVANCE TO PARKINSON'S DISEASE

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MPTP damages dopaminergic neurons in the substantia nigra pars compacta as seen in Parkinson's disease (PD). We previously demonstrated that the pro-apoptotic protein Bax plays a pivotal role in MPTP-induced neurodegeneration by activating the mitochondrial-dependent apoptotic pathway (i.e. release of mitochondrial cytochrome c to the cytosol). It has been suggested that the release of cytochrome c in this model, as well as in PD, may result directly from the decreased activity of mitochondrial complex I and the subsequent inhibition of mitochondrial respiration that occur in both MPTP-intoxicated animals and PD patients. In order to test this hypothesis, we incubated isolated non-synaptosomal mouse brain mitochondria with different concentrations of MPP⁺ and rotenone, two complex I inhibitors that cause experimental parkinsonism in rodents, and we monitored, in parallel, oxygen consumption, mitochondrial membrane potential, reactive oxygen species (ROS) production and release of cytochrome c. At the different concentrations tested (which resulted in a 25 to 100% inhibition of mitochondrial respiration), we did not observe any release of cytochrome c at any time. However, at concentrations that produce ROS, inhibition of complex I potentiated the release of cytochrome c induced by Bax or BH3 domain-only recombinant proteins. By assessing the ratio of ascorbate-driven respiration over TMPD-driven respiration, we showed that complex I inhibition increases the amount of cytochrome c freely available in the mitochondrial intermembrane space. Because cytochrome c is attached to the inner mitochondrial membrane by cardiolipin, this interaction must be first disrupted to generate a soluble pool of this protein in the intermembrane space, which can occur by peroxidation of cardiolipin by ROS. In agreement with this, we showed by HPLC that at concentrations that produce ROS, inhibition of complex I induces a significant increase in oxidized cardiolipin (CLOOH) content in isolated brain mitochondria. In summary, our results suggest that ROS induced by complex I inhibition results in lipid peroxidation, which disrupts the interaction between cytochrome c and cardiolipin, thus facilitating the release of cytochrome c by pro-apoptotic molecules.

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THE PYRUVATE DEHYDROGENASE COMPLEX AS A TARGET OF OXIDATIVE STRESS DURING CEREBRAL ISCHEMIA/REPERFUSION

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The pyruvate dehydrogenase complex (PDHC) catalyzes the oxidative decarboxylation of pyruvate, a product of glycolysis. Previous research indicates that PDHC may serve as a target for toxic reactive oxygen species (ROS), but it is unknown whether reactive nitrogen species (RNS), such as peroxynitrite (ONOO⁻) can also inactivate the enzyme. The purpose of this study was to investigate the hypothesis that the PDHC serves as a target for oxidative stress during cerebral ischemia/reperfusion. Assays of enzymatic activity performed on isolated PDHC enzyme or rat brain mitochondria were used to determine the effects of ONOO⁻ on PDHC activity. PDHC activity decreased in a dose- and time-dependent manner in the presence of increasing amounts of the ONOO⁻ generating system, 3-morpholinosynadmine (SIN-1). These findings were associated with increased nitrotyrosine (NT) immunoreactivity of the PDHC complex. Immunohistochemical analysis of canine hippocampal PDHC after 10 minutes of global ischemia, followed by 2 hours of reperfusion shows a decrease in PDHC staining that was significantly more pronounced in animals that were resuscitated with 100% O₂ (hyperoxic), compared to room air (normoxic). Conversely, NT immunoreactivity was 68.5% higher in animals resuscitated under hyperoxic conditions. A slight reduction in PDHC immunoreactivity was also observed with immunoblots performed on homogenates or isolated mitochondria from both cortical and hippocampal samples following resuscitation under hyperoxic conditions. Increased oxidative stress associated with ischemia/reperfusion may be responsible for decreased PDHC activity which can limit cerebral energy metabolism and contribute to the poor neurological outcome that has been documented in animals that undergo cardiac arrest followed by 24 hours resuscitation under hyperoxic conditions. (Supported by AHA 0215331U to E.M. and NIH NS34152 to G.F.)



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PYRUVATE - A JANUS-FACED MOLECULE IN THE FREE RADICAL MIRROR.

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Pyruvate and lactate have a central role in the glia – neuron communication. The purpose of the present study was to investigate the effect of pyruvate on Reactive Oxygen Species (ROS) formation and elimination in isolated nerve terminals (synaptosomes). Using pyruvate as a respiratory substrate the rate of ROS-formation increased by 80 % as compared to the substrate-free conditions. This increment in ROS production was significantly higher than that induced by glucose and lower than that with α -ketoglutarate. Mitochondrial aconitase is extremely sensitive to free-radicals and widely used to detect ROS formation. Contrary to our expectation, aconitase activity remained preserved in synaptosomes incubated with pyruvate in spite of the enhanced ROS production. Detergent treatment of synaptosomes decreased aconitase activity dramatically and pyruvate was not able to prevent the inactivation. If substrates of aconitase e.g. citrate or isocitrate were present during the incubation with detergent, aconitase activity was partially preserved. These data indicate that pyruvate has to be metabolized in order to protect aconitase. Part of the protecting effect of pyruvate can be attributed to the formation of citrate and isocitrate. In addition, pyruvate significantly increased the rate of organic hydroperoxide (t-BOOH) elimination, indicating that active mitochondrial metabolism is necessary for the detoxification of hydroperoxides. Recently we and Starkov et al., (submitted) have shown that isolated pyruvate dehydrogenase (PDHC) is able to form superoxide and H_2O_2 . Here we show that when all the substrates and cofactors of the enzyme were present, the NADH/NAD⁺ ratio determined the rate of H_2O_2 production similarly to that observed earlier with α -KGDH (Tretter and Adam-Vizi submitted). The higher the NADH/NAD⁺ ratio the higher was the rate of H_2O_2 production. It is also shown that with isolated PDHC both H_2O_2 production and the catalytic function of the enzyme were activated by Ca^{2+} . Given the NADH/NAD⁺ ratio as a regulator of H_2O_2 production by PDHC, it is suggested that production of ROS could be significant not only in the respiratory chain but also in the PDHC and KGDH when oxidation of NADH is impaired. On the other hand metabolism of pyruvate results intermediates protecting against ROS-mediated damage.

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PDGF ALTERS MITOCHONDRIAL DYNAMICS IN OLIGODENDROCYTE PROGENITOR CELLS (OP CELLS) IN CULTURE

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We recorded mitochondrial movement and dynamics in OP cell processes using organelle specific fluorescent dyes. OP cells isolated from newborn rats were maintained in primary culture either under growth-arrested conditions (N1 medium) or in proliferative state with N1 medium containing PDGF. Cells were stained with Mitotracker Red-CMXROs or Mitofluor Green and were observed by time-lapse microscopy over a 2 hour period. Almost all mitochondria were motile and showed dynamic shape changes, fusions and divisions. Some motile mitochondria were found to change direction from orthograde to retrograde or vice versa within processes. We observed moving mitochondria fusing with another to make a single organelle as well as mitochondria splitting into two separate organelles. Often, mitochondria showed shape changes from rounded morphology to elongated "worm-like" shapes and back. Mitochondrial motility and shape changes were consistently enhanced in proliferating OP cells with PDGF compared with growth arrested cells. In PDGF treated cells, 100% of the mitochondria were seen to move during the experiment, whereas less than 30% of the mitochondria were motile in cells in N1 medium. Mitochondria moved along processes both away from the soma as well as towards the cell body and the velocity of this movement was dramatically higher in PDGF treated cells. The velocity in N1 cultured cells was $0.48 \pm 0.25 \mu\text{m}/\text{min}$ (mean \pm SD) and in PDGF it increased to $1.77 \pm 0.93 \mu\text{m}/\text{min}$. This data suggests that PDGF- α receptor activation signals mitochondria in OP cells as they become proliferative and progress towards differentiation.



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MITOCHONDRIA MOVEMENTS ARE IMPAIRED FOLLOWING MODERATE HYPOXIA AND ACUTE NITRIC OXIDE EXPOSURE IN CULTURED CORTICAL NEURONS FROM FETAL RATS

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Background/Aim: Cortical nitric oxide (NO) production increases during hypoxia/ischemia-reperfusion in the immature brain and is associated with neurotoxicity and mitochondrial dysfunction. Mitochondria movement and redistribution within the cell is critical to normal neuronal function. However, the effect of hypoxia on mitochondria movement in neurons is not well known. The present study tests the hypothesis that hypoxia impairs mitochondria movement in cortical neurons via nitric oxide (NO)-mediated pathways. **Methods:** Cultured cortical neurons (DIV 10-14), isolated from E19 Sprague-Dawley rat fetuses were exposed to hypoxia for 6h followed by 0, 1, 2 and 24h re-oxygenation or to 500 μ M of the NO donor, diethylenetriamine/nitric oxide adduct (DETA-NO) for 1, 2 and 3h and compared to normoxic controls. N^G-nitro-L-arginine methyl ester (L-NAME, 10 μ M), a non-specific NO synthase (NOS) inhibitor was used prior to hypoxia exposure to determine NO specificity. Movement of mitochondria labeled with Mitotracker CMX-Ros (20nM) was analyzed in dendrites using time-lapse digital video microscopy. **Results:** Cortical neurons exposed to hypoxia-reoxygenation demonstrated a 46% decreased velocity from 0.177 \pm 0.02 μ m/sec at 1h of re-oxygenation as compared to 0.385 \pm 0.01 μ m/sec in controls (p <0.001). Mitochondria movement velocity progressively recovered to 68 and 94% of control values at 2 and 24h of re-oxygenation, respectively: (0.260 \pm 0.02 μ m/sec at 2h and 0.363 \pm 0.03 μ m/sec at 24h). Similarly, continuous exposure to 500 μ M DETA-NO resulted in decrease mitochondria velocity at all tested time points: 41%: 0.158 \pm 0.01 μ m/sec at 1h; 45%: 0.175 \pm 0.01 μ m/sec at 2h; and 40%: 0.154 \pm 0.01 μ m/sec at 3h (p <0.001 vs. control). Treatment with L-NAME prior to hypoxia exposure partially reversed the decrease in mitochondria velocity by 68%: 0.299 \pm 0.02 μ m/sec (p =NS vs control). **Conclusions:** Both hypoxia and NO exposure result in impaired mitochondria movement in cultured cortical neurons. The effect of hypoxia on mitochondria movement can be partially prevented during at least the first 2 hours of reoxygenation by the use of NOS inhibitor, indicating NO-mediated pathways. We speculate that impaired mitochondria movement may contribute to neuronal cell death during transient hypoxia.

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INTRACELLULAR CALCIUM CONTROLS MITOCHONDRIAL MORPHOLOGY

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Mitochondria function in cellular ATP production, Ca²⁺ homeostasis, ROS generation, and apoptosis. Mitochondria are dynamic organelles that undergo constant fission and fusion as well as trafficking along the cytoskeleton. Although this rapid change of shape and number, namely mitochondrial dynamics are thought to participate in temporal and spatial regulation of mitochondrial function, how the mitochondrial dynamics are controlled is poorly understood. Since Ca²⁺ is a key regulator of mitochondrial function, this study tested the hypothesis that intracellular Ca²⁺ is a regulatory component controlling mitochondrial morphology. Application of thapsigargin (TG) or cyclopiazonic acid (CPA) to inhibit SERCA induced a concentration-dependent transient increase of cytosolic Ca²⁺ concentrations that returned to basal levels within 15 minutes in a rat liver cell line. Upon the same treatment, mitochondria became fragmented within 5 minutes. Remarkably, fragmented mitochondria returned to the initial tubular morphology within 2 hours, indicating a reversible and transient process. Quantitation of mitochondrial morphology demonstrated that the degree of mitochondrial fragmentation correlates well with the magnitude of cytosolic Ca²⁺ transients induced by TG or CPA. Although the normal mitochondria morphology was restored during these treatments, a second phase of mitochondrial fragmentation occurred in 6-8 hours, which led to the apoptotic cell death as indicated by chromatin condensation. These results suggest that there are two separate mechanisms for controlling mitochondrial morphology and the cytosolic Ca²⁺ transient may mediate the first, reversible mitochondrial fragmentation. Importantly, this TG-induced mitochondrial fragmentation was blocked in cells expressing dominant negative DLP1, the mitochondrial fission protein. Surprisingly, chelating intracellular Ca²⁺ by BAPTA-AM also caused mitochondrial fragmentation, which, was again prevented with the expression of the dominant negative DLP1. Unlike TG treatment, however, the BAPTA-induced mitochondrial fragmentation was not reversible and cells rapidly underwent apoptosis. In conclusion, these results provide evidence that intracellular Ca²⁺ is a crucial component that regulates mitochondrial morphology presumably through its effect on the mitochondrial fission machinery.



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PROLONGED Zn^{2+} EXPOSURE CAUSES MITOCHONDRIAL FRAGMENTATION AND AGGREGATION IN A STABLE NEURONAL CELL LINE EXPRESSING FLUORESCENT MITOCHONDRIA

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Elevated intracellular free zinc ($[Zn^{2+}]_i$) is a potent neurotoxin. As a mitochondrial toxin, $[Zn^{2+}]_i$ has been previously shown to disrupt mitochondrial energy production and increase reactive oxygen species. Importantly, mitochondria are dynamic entities, and emerging evidence suggests that changes in mitochondrial morphology and motility in response to injury may compromise neuronal energy distribution. We hypothesized that toxic $[Zn^{2+}]_i$ can alter mitochondrial morphology and cellular localization. To visualize mitochondria in live cells, we have stably expressed mitochondrially-targeted enhanced yellow fluorescent protein (mt-eYFP) in the HT22 neuronal cell line. Upon exposing cells to pathophysiological levels of Zn^{2+} (3 μ M) in the presence of the ionophore sodium pyrithione (20 μ M), we observed mitochondrial fragmentation as early as 2 hours after treatment. Using the cell viability indicator Hoechst-33342, nuclear condensation was observed as early as 1 hour after Zn^{2+} exposure, depicting a linear increase in Zn^{2+} -mediated toxicity over time. In addition, using an anti-cytochrome c antibody, we found that cytochrome c staining co-localizes with mitochondrial fluorescence 2 hours post Zn^{2+} exposure, but this staining disappears 4 hours after treatment. From these series of experiments, we conclude that there is indication that nuclear condensation occurs before mitochondrial fragmentation, which occurs prior to cytochrome c release. We are currently investigating other parameters in relation to these changes by: (1) blocking caspase activation using caspase inhibitors, (2) preventing mitochondrial permeability transition using cyclosporin A, and (3) examining changes in mitochondrial membrane potential over various time points after Zn^{2+} treatment. This study presents evidence for a sequence of events relating Zn^{2+} -induced changes in mitochondrial morphology to cell death. *Supported by NIH grant (IJR) and AHA fellowships (LMM and GLR).*

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ASTROCYTES PROTECT NEURONS FROM ETHANOL-INDUCED OXIDATIVE STRESS AND MITOCHONDRIALLY-MEDIATED APOPTOTIC DEATH

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Studies in this laboratory have illustrated that: (1). In utero ethanol (E) exposure generates apoptotic death of neurons concomitant with oxidative stress (OS) in fetal rat brain, (2). This is mitochondrially-mediated, and (3). The enhanced apoptosis may be connected to 4-hydroxynonenal (HNE) production in mitochondria (M). In cultured fetal cortical neurons (N), E elicited increased levels of reactive oxygen species (ROS) within minutes, followed by increased HNE, release of cytochrome C, and activation of caspase-3. Onset of apoptotic cell death was reflected by increased Annexin V binding ($49\% \pm 0.7$, $60\% \pm 1.6$, $146\% \pm 5.6$ and $269\% \pm 1.76$ at 2, 6, 12 and 24 hours of E treatment, respectively, mean \pm S.E.M., $p < 0.05$) and DNA fragmentation ($38\% \pm 1.6$ and $53\% \pm 1.8$ by 12 and 24 hours of E exposure, respectively, mean \pm S.E.M., $p < 0.05$). This was associated with reduced glutathione (GSH) content of $45\% \pm 13$ to $72\% \pm 15$ by one hour of E exposure (2.5 and 4 mg/ml, respectively) and $73\% \pm 8$ to $84\% \pm 5.2$ by two hours.

The ability of astrocytes (A) to protect N from E-related OS was assessed using co-cultures of neonatal cortical A and neurons. The presence of A prevented the initial E-related decrease in neuron GSH content, protected N from decreased viability, and reversed the increased ROS, Annexin V binding, and DNA fragmentation. E (2.5 and 4.0 mg/ml) increased GSH efflux ($27\% \pm 12.4$ and $39\% \pm 15.2$, respectively, $p < 0.05$) from A while enhancing the activity of γ glutamyl transpeptidase (GGT) ($25\% \pm 0.6$, $35\% \pm 1.3$, and $44\% \pm 1.2$, $p < 0.05$). Thus, the mechanism by which A protect N from E-mediated apoptosis may be supplementation of cellular GSH. The efficacy of this process is enhanced by an E-related increase of astrocyte extrusion of GSH and its conversion to the CysGly dipeptide, a precursor for neuronal cysteine.

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PROBING FOR TRP CHANNEL FUNCTIONS ON CORTICAL NEURONS IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

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Channels of the transient receptor potential (TRP) family debut in the field of intracellular calcium research during the past 2 decades. Recently, there is an explosion in experimental findings relating TRP channels to a number of physiological and pathological processes in non-excitable cells. However, they are also prominently expressed in excitable cells, such as in neurons. We explored the conditions in which we could demonstrate the contribution –or lack thereof– of TRP channels in $[Ca^{2+}]_i$ homeostasis in cultured cortical neurons. As TRP proteins are mainly non-specific cation channels, a basal Me^{2+} entry protocol was employed, in which neurons are deprived of extracellular calcium (nominally Ca^{2+} -free medium or <100 nM $[Ca^{2+}]_e$ in the absence of SERCA inhibitors) for 10 min, followed by reintroduction of a cation (Ca^{2+} , Ba^{2+} , Sr^{2+} , Mn^{2+}) to the perfusing medium. We demonstrate a robust basal Me^{2+} entry (BME), in which neurons exhibit a sharp rise of $[Me^{2+}]_i$ followed by a decay to baseline values, which is mitigated by extracellular Mg^{2+} and inversely correlated to $[Ca^{2+}]_e$. BME was moderately affected by a concomitant inhibition of ligand- and voltage- gated cation channels, but was strongly dependent on the mitochondrial membrane potential; when in situ neuronal mitochondria were depolarized by rotenone plus oligomycin, neurons exhibited much smaller BME, and virtually no return to baseline values throughout the presence of the extracellular cation. BME was aggravated by the concomitant presence of an oxidative stress caused by exogenous addition of H_2O_2 (250 μ M), increasing peak amplitude and eliminating the postexposure $[Me^{2+}]_i$ decay. Prior abolition of mitochondrial membrane potential partially alleviated the deleterious effect of the oxidative stress on BME. Currently, the only putative ligand of a subset of TRP channels (TRPC3/6/7) identified is diacylglycerol. Addition of the membrane-permeant diacylglycerol analogue 2-Acetyl-1-oleoyl-sn-glycerol (OAG) caused $[Ca^{2+}]_i$ oscillations, that were abolished by La^{3+} but not by inhibition of protein kinase C. These results together with future attempts to silence the expression of individual members of the TRP family, will elucidate their contribution in neuronal $[Ca^{2+}]_i$ homeostasis. Supported by NIH grant NS34152 to G.F.

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TRP CHANNEL BLOCKERS MITIGATE GLUTAMATE-INDUCED DELAYED CALCIUM DEREGLATION IN CULTURED CORTICAL NEURONS

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Exposure of neurons in culture to excitotoxic levels of glutamate leads to a latent, irreversible $[Ca^{2+}]_i$ rise termed delayed calcium deregulation. In spite of a gamut of pathways excluded, the molecular counterparts responsible for the secondary Ca^{2+} rise have never been identified. We report that the delayed Ca^{2+} entry is diminished by 2-APB ($IC_{50} = 66 \pm 5 \mu$ M) and La^{3+} ($IC_{50} = 7.2 \pm 3 \mu$ M), both known to inhibit transient receptor potential (TRP)- and store-operated Ca^{2+} (SOC)-channels. However, cortical neurons failed to exhibit SOC entry. In basal Ca^{2+} entry experiments, La^{3+} and 2-APB modulated the rapid rise in $[Ca^{2+}]_i$ caused by exposure of neurons to Ca^{2+} after preincubating in a calcium-free medium. This basal Ca^{2+} influx was diminished by extracellular Mg^{2+} , but not aggravated by preempting intracellular stores. These results indicate that 2-APB and La^{3+} influence non-store-operated Ca^{2+} influx in cortical neurons and that this route of Ca^{2+} entry is involved in glutamate-induced delayed Ca^{2+} deregulation.

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RECEPTOR-INDEPENDENT EFFECTS OF ANTI-DIABETIC PPAR γ AGONISTS ON ASTROCYTE METABOLISM: CYTOPROTECTIVE AND CYTOTOXIC ACTIONS

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An increasing number of studies have demonstrated that agonists of the peroxisome proliferator activated receptor gamma (PPAR γ) exert neuroprotective effects in animals models of neurological disease including MS, AD, PD, and stroke. Although these effects are due in part to anti-inflammatory actions of PPAR γ activation, some responses are more pronounced with lower affinity rather than higher affinity agonists, suggesting non-receptor mediated effects. We tested the direct actions of several PPAR γ agonists on astrocyte metabolism. Treatment with thiazolidinedione (TZD) agonists, but not non-TZD agonists, increased astrocyte glucose uptake and metabolism. In isolated mitochondria, TZDs inhibited pyruvate driven, but not glutamate driven state III respiration; while in astrocytes, incubation with TZDs caused mitochondrial hyperpolarization. Further studies on isolated mitochondria indicate that TZDs do not inhibit succinate/rotenone driven respiration; or CACT activity. TZDs did not inhibit PDH activity of either intact or ruptured mitochondria or of purified PDH. After overnight treatment with TZDs, astrocytes were less susceptible to cell death induced by hypoglycemia, or by staurosporin. In contrast, 24 to 48 hr treatment of the mouse glioma cell line GL26 induced cell death. These data demonstrate that TZDs directly influence astrocyte mitochondrial function leading to changes in glucose metabolism. In astrocytes, these changes result in cytoprotection possibly by activation of compensatory mechanisms, while in transformed cells such mechanisms do not come into play and cell death ensues. Since several TZDs are already FDA-approved (for treatment of type-2 diabetes), they are good candidates for testing in clinical trials of neurological disease. *This work was supported by grants from the National MS Society and Takeda Pharmaceuticals.*

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A NEW TECHNIQUE FOR ISOLATION OF FUNCTIONALLY AND MORPHOLOGICALLY INTACT MITOCHONDRIA FROM PRIMARY NEURONAL AND ASTROCYTE CELL CULTURE

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Heterogeneity of brain mitochondria can be originated from the heterogeneity of brain cell populations (mitochondria from neurons versus glia). In order to study the cell specific mitochondria one need to isolated these organelles from primary neuronal or glial cell cultures. We have developed a new method for isolation of mitochondria from cell culture by using a nitrogen cavitation technique followed by Percoll gradient centrifugation. The mitochondria obtained by our isolation procedure displayed an excellent respiratory functions and morphological integrity. Mitochondria isolated from astrocytes had a respiratory control ratio (RCR) of about 9 and the RCR of neuronal mitochondria was about 11. The difference observed in RCR values was mainly due to the lower rates of respiration in state 4 found in neurons as compared to that found in astrocytes. This suggests a better coupling of oxidative phosphorylation in neuronal mitochondria. Electron microscopic examination revealed that the mitochondria obtained by our technique are morphologically intact and also that the matrix morphology of neuronal and glial mitochondria is not identical. The organization of matrix cristae in neuronal mitochondria has a parallel alignment. The cristae in astrocytes mitochondria have random orientation. Furthermore, mitochondria originated from astrocytes are highly enriched by peripheral benzodiazepine receptor (PBR) and by pyruvate carboxylase (PC) when compared to neuronal one. Our data suggest that the combination of nitrogen cavitation technique and gradient centrifugation yields to mitochondria displaying exceptional functional and morphological integrity and that there are functional and morphological differences between the two types of mitochondria. This study was supported by AHA 0256359 to T.K. and NIH NS34152 to G.F.



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REGION SPECIFIC, AGE-RELATED, ALTERATIONS IN MITOCHONDRIAL RESPONSES TO INCREASING CALCIUM

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An age-related decline in cellular Ca^{2+} regulation and increased production of reactive oxygen species (ROS) may contribute to the pathogenesis of late-onset neurodegenerative disorders. The cause of these alterations is often attributed to impaired mitochondrial function yet few studies have directly examined the properties of mitochondria isolated from various regions of the aged brain. The purpose of this study was to examine mitochondrial bioenergetics, Ca^{2+} -buffering and ROS production in mitochondria isolated from male Fischer 344 rats ranging in age from 3 to 24 months. Furthermore synaptic and non-synaptic mitochondrial fractions were examined for age-related changes in function. Mitochondria isolated from the cortex of the 24 month rat brain produced significantly greater ROS levels and swelled more readily in response to increasing Ca^{2+} loads compared to mitochondria isolated from younger animals. These age-related differences were not observed in mitochondria isolated from cerebellum. The increased swelling is indicative of opening of the mitochondrial permeability transition pore indicating impaired Ca^{2+} buffering/cycling in aged animals. In ongoing studies, our preliminary results suggest that synaptic mitochondria, which are predominately neuronal in origin, swell in response to lower Ca^{2+} concentrations than non-synaptic mitochondria, regardless of age. Together, these results demonstrate region specific, age-related, alterations in mitochondrial homeostasis. This research was supported by a grant from the Alzheimer's Association (JWG) and by NIH grants AG00264 (MRB), AG10836 (JWG), and NS 048191 (PGS).

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NEURON MITOCHONDRIAL FUNCTION DECLINES WITH AGE AND STRESS: REJUVENATION WITH ESTROGEN OR MITOSIS

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The mitochondrial oxyradical theory of aging is gaining increased support from in situ studies that often do not identify the source of the deficits. Is this aging caused by systemic hormones or intrinsic to aging mitochondria in neurons? The intrinsic hypothesis of cell aging is supported by our findings with isolated neurons in which there is increased susceptibility with age to glutamate and A-beta toxicity, two endogenous toxins important to age-related neurodegenerative disease (Brewer, 1998). For neuron-specific function, we have established culture techniques that allow us to compare neurons isolated from embryonic, middle-age (12 month) and old (24 month) rat hippocampus (Brewer, 1997). We monitored mitochondrial cytochrome oxidase activity (COX), cardiolipin with nonyl-acrydine orange (NAO), with mitotracker-red and immunostain for cytochrome C, fluorescent NADH, glutathione and respiration. Cytochrome C immunostain and mitotracker red stained equal numbers of mitochondria per cell for middle-age and old neurons, compared to higher numbers per embryonic neuron. In contrast to mitochondrial numbers, COX activity/cell declined sharply with age of the neurons: levels of old neurons were reduced by 30% of those of middle-age neurons. Similarly, NAO stain for mitochondria was 40% lower for old neurons compared to middle-age neurons. By addition of FGF2 and low density passage, old neurons divide in culture (Brewer, 1999). COX activity in these dividing old neurons was equivalent to that of middle-age neurons. Age-related reductions in glutathione levels were exacerbated by exposure to glutamate. NADH levels and respiration in old neurons were normal, but showed dramatic deficits in response to glutamate. In addition to restoring youthful calcium dynamics, treatment of old neurons with 17-beta-estradiol restored NAO staining levels/cell to those of middle-age neurons and provided full neuroprotection from glutamate and A-beta toxicity. These results show that mitochondrial function is impaired in old neurons, which correlates with increased susceptibility to toxic stress, but function can be restored by treatment with FGF2 or estradiol.



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MITOCHONDRIAL AGING IN THE CANINE BRAIN: IMPLICATIONS FOR MITOCHONDRIAL BIOENERGETICS AND HOMEOSTASIS

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Mitochondria are the primary source of ATP in cells of the CNS. During the process of aerobic metabolism reactive oxygen species (ROS) can be formed as electrons leak from the mitochondrial electron transport system (ETS). ROS have the capacity to damage mitochondrial proteins, lipids and mtDNA, which could impair mitochondrial ATP production and further increase electron leak. The present study assesses whether cumulative mitochondrial oxidative damage occurs as a function of age and whether dietary antioxidants have an effect on mitochondrial bioenergetics in the aging canine brain. Mitochondria were isolated by biopsy from the parietal cortex of young (n = 5; mean = 3.4 yrs) or aged (n = 18; mean = 10.69 yrs) beagle dogs prior to euthanasia. The young dogs and half the aged animals had been maintained on a standard diet (SD). The remaining aged dogs had been maintained on an antioxidant-enriched (AO) diet for 2.4 - 2.8 years. Several parameters of mitochondrial bioenergetics were assessed including mitochondrial respiration, ROS production and oxidative damage in individual animals. The results demonstrate that mitochondria isolated from young dogs had significantly greater complex I-driven ETS activity compared to either group of aged animals. Complex II-driven respiration in mitochondria isolated from young was significantly increased compared to aged control dogs but not significantly different than aged AO-animals. AO-treated aged animals demonstrated a significant increase in complex I-driven respiration compared to SD counterparts, but no difference was measured in complex-II driven respiration. Mitochondrial ROS production was significantly increased in aged animals compared to young dogs and mitochondria isolated from aged AO-treated animals produced significantly less ROS than SD counterparts. Mitochondrial protein oxidation was also significantly increased in aged animals compared to young dogs. Interestingly, AO aged animals had significantly higher levels of mitochondrial protein carbonyls compared to SD counterparts. These data indicate that mitochondrial bioenergetics is comprised as a function of age in the canine brain. Furthermore the decline in mitochondrial function correlates with an age-dependent increase in ROS production and oxidative damage to mitochondrial proteins. Importantly, increasing dietary antioxidants maintain mitochondrial homeostasis in the aged brain.

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UP-REGULATION OF MITOCHONDRIAL GENES IN THE BRAINS OF AMYLOID PRECURSOR PROTEIN TRANSGENIC MICE AND IN ALZHEIMER'S DISEASE PATIENTS: IMPLICATIONS FOR EARLY MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE DAMAGE

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The pathogenesis of Alzheimer's disease (AD) has not been established with certainty, but abnormalities in the processing of the amyloid precursor protein (APP) have been implicated. Our objective was to determine genes that are critical for early cellular changes in AD progression. We investigated an established APP transgenic mouse model for gene expression profiles at 3 stages of disease progression: long before, immediately before, and after the appearance of A β plaques. The regional distribution of gene expression changes was examined with fluorescent-labeled *in situ* hybridization and immunohistochemistry techniques in brain tissue from APP mice and wild-type mice. The APP-mouse gene-expression profile was distinguished by the up-regulation of genes related to OXPHOS of mitochondrial energy metabolism compared to wild-type mice. This difference was evident by 2 months of age and became more pronounced at 5 and 18 months. Results from *in situ* hybridization analysis of the mitochondrial-encoded gene - ATPase-6, and programmed cell death gene 8, revealed that pyramidal neurons in brain slices from the hippocampus and cerebral cortex are up-regulated in APP mice. Results from double-labeling *in situ* hybridization of mitochondrial gene - ATPase-6, and immunofluorescence analysis of 8-hydroxyguanosine suggest that only selectively over-expressed neurons with ATPase-6 undergo oxidative damage in APP mice. To confirm these changes in AD patients, using quantitative RT-PCR techniques, we investigated mitochondrial-encoded genes in brain specimens from AD patients and control subjects. Our RT-PCR analysis revealed a down-regulation of mitochondrial genes in complex I in AD brain specimens. Contrary to the down-regulation of genes in complex I, complexes III and IV showed increased mRNA expressions in the brain specimens of both early and definite AD patients, suggesting a great demand on energy production. Based on these results, we propose that mitochondrial energy metabolism is impaired by the expression of mutant APP and/or A β , and that the up-regulation of mitochondrial genes represents a compensatory response.



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MITOCHONDRIAL DYSFUNCTION MAY PLAY A ROLE IN THE DEVELOPMENT OF NEURODEGENERATIVE DISEASE IN MICE WITH A TARGETED DELETION OF IRP2

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Oxidative stress has been implicated in the genesis of neurodegenerative disorders including Parkinson disease, Alzheimer disease and amyotrophic lateral sclerosis. Iron is an etiologic agent in oxidative stress and accumulations of iron are frequently observed in regions of the brain affected by neurodegenerative disorders. In mammalian cells iron metabolism is tightly regulated by two distinct but highly homologous iron regulatory proteins, IRP1 and IRP2, which interact with mRNA transcripts that contain stem-loop structures called iron responsive elements (IREs). When cells are depleted of iron, IRPs bind IREs with high affinity, resulting in increased mRNA stability for some transcripts, such as transferrin receptor, or translational inhibition of some transcripts, such as ferritin. Mice with a targeted deletion of the gene encoding IRP2 develop a progressive neurodegenerative disorder in adulthood. Symptom onset at 6 months of age is characterized by ataxia, bradykinesia, tremor, and severe weakness. There is a widespread axonopathy in central and peripheral nervous systems, loss of neuronal cell bodies in the substantia nigra and cerebellar cortex and swollen dystrophic neurites and axonal spheroids in cerebellar white matter. Increased accumulation of ferric iron in neurons and oligodendrocytes in discrete regions of brain precedes neurodegenerative findings by many months with parallel increase in brain ferritin levels and decrease in levels of transferrin receptor. Our preliminary results suggest mitochondrial dysfunction in IRP2 knockout mice. We find elevations of glycolytic enzymes and decreased levels of mitochondrial RNA transcripts relative to wild type controls. We plan to further evaluate possible mitochondrial dysfunction in IRP2 knockout mice to determine if mitochondrial biogenesis or respiratory chain function is impaired and the role this dysfunction may play in the etiology of the neurodegenerative disorder they develop.

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STUDY OF Ca^{+2} HOMEOSTASIS AND BEHAVIOR IN THE NEURODEGENERATIVE PROCESS OF HUNTINGTON DISEASE

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During cell signaling Ca^{+2}_c overload can occur. One example is the mechanism of excitotoxicity which is referred to deleterious effect on neuronal cells by relatively high concentration of glutamate (Glu). In this case the increase in Ca^{+2}_c can be buffered by several mechanisms as mitochondrial Ca^{+2}_m uptake which is not only important for Ca^{+2}_c buffering, but also for the ATP production. However, the accumulation of Ca^{+2}_m can induce changes in mitochondrial pH, increase in ROS production, opening of permeability transition pore (PTP) and Ca^{+2}_m release. These events may cause mitochondrial dysfunction and can play a significant role in several neurodegenerative disorders as Huntington Disease (HD). The purpose of this work was to investigate Ca^{+2} alterations and mitochondrial dysfunction, as well as behavioral changes during the disease development. This study was performed in transgenic mice (TGn) for HD (R6/1 strain) with different ages. Ca^{+2} homeostasis was studied in brain slices of TGn and control mice loaded with Fura-2-AM or Fluo-3-AM. The experiments were performed with high resolution fluorescence microscopy. Brain slices were stimulated with Glu (1mM) and KCl (200 mM) and fluorescence was extracted and analyzed from digital images. Behavioral studies were performed every third week between the 16th and 40th weeks of age. The parameters of locomotion and immobility were measured. In brain slices stimulated with Glu TGn mice showed a significant increase in Ca^{+2}_c levels (1.07 ± 0.18) in relation to the control (0.57 ± 0.23). This increase in Ca^{+2}_c occurred as a wave that propagated from cortex to the striatum and the magnitude of this response was dependent on the brain region. Ca^{+2}_c was significantly higher in TGn than in control animals in the striatum (1.15 ± 0.18 X 0.62 ± 0.29) and cortex (1.21 ± 0.16 X 0.57 ± 0.21). Behavioral studies showed that TGn mice presented a decrease in general activity between 34^a and 40^a weeks of age when compared with controls. The locomotion in the 37^a week was 101.1 ± 34.6 in the TGn and 78.4 ± 20.8 in the control. These results suggest that alterations in Ca^{+2}_c handling in transgenic mice might be associated with the neurodegenerative process in Huntington disease. Support : FAPESP and CNPq



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PATHOLOGICAL CONSEQUENCES OF A NOVEL MISSENSE MUTATION IN THE MITOCHONDRIAL CYTOCHROME B GENE

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Paracrystalline inclusions were found in a young male endurance athlete who had a very high respiratory exchange ratio during steady-state exercise and an unusually low aerobic capacity given his training history. Direct sequencing of mitochondrial DNA (mtDNA) coding regions revealed a novel missense mutation (G15497A) resulting in a glycine → serine conversion at a highly conserved site in the cytochrome b gene in the subject. Cybrids, prepared by fusion of the subject's platelets with either U87MG p^o or SH-SY5Y p^o cells, generated higher basal levels of reactive oxygen species (ROS), had a lower ATP content and were more sensitive to oxygen and glucose deprivation and peroxynitrite generation compared to control cybrids with wild-type mtDNA. Cell survival under both conditions was significantly enhanced as a result of co-treatment with 50 mM creatine monohydrate (CM) and 10 µg/ml of CoQ10. The subject was also treated with CM (10 g/d) for a period of five weeks and had a repeat muscle biopsy of the ipsilateral vastus lateralis, which revealed no paracrystalline inclusions. After discontinuing CM supplementation for three months and increasing exercise training for two months, paracrystalline formation and electron densities were observed again. The results suggested that this missense G15497A mtDNA mutation might have contributed to the formation of paracrystalline inclusions by increasing the cellular vulnerability to metabolic and oxidative stress.

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DEVELOPMENT AND INITIAL CHARACTERIZATION OF XENOMITOCHONDRIAL MICE

I.A. Trounce, M. McKenzie, C.A. Cassar, C.A. Ingraham, C.A. Lerner, D.A. Dunn, C.L. Donegan, K. Takeda, W.K. Pogozelski, R.L. Howell and C.A. Pinkert
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Xenomitochondrial mice harboring trans-species mitochondria on a *Mus musculus domesticus* (MD) nuclear background were produced. Initially, methods for mitochondrial isolation and inter-species transfer were devised using mitochondria injection into mouse ova. Transmitochondrial founders were obtained and germ-line transmission of the heteroplasmic state was observed in maternal lineages (Irwin *et al.*, *Transgenic Res.*, 1999; Pinkert and Trounce, *Methods*, 2002). A second route for model development involved hybrid fusions with *Mus spretus* (MS), *Mus caroli* (MC), or *Mus dunni* (Mdu) mitochondrial donor cytoplasts and rhodamine 6-G treated CC9.3.1 or PC4 ES cells. The selected donor backgrounds reflected increasing evolutionary divergence from MD mice and the resultant mitochondria:nuclear mismatch targeted a graded respiratory chain defect. Homoplasmic (MS, MC and Mdu) and heteroplasmic (MC) cell lines were injected into MD ova, and liveborn chimeric mice were obtained (MS:MD 18/87, MC:MD 6/46, and Mdu:MD 29/134 founder chimeras, respectively). Founders exhibited up to 99% coat color chimerism. Males were infertile or did not transmit the cytoplasm-derived mitochondria to offspring. Seven MS:MD, 1 MC:MD, and 11 Mdu:MD chimeric founder females were mated with wild-type MD males and 18 of 19 (95%) were fertile. Of fertile females, only one chimeric MS:MD (1% coat color chimerism) and three chimeric Mdu:MD females (80-90% coat color chimerism) produced homoplasmic offspring with low efficiency (6/117; 5%). Four male and two female offspring were homoplasmic for the introduced mitochondrial backgrounds. Yet, only three male offspring proved viable (surviving beyond one day of age; McKenzie *et al.*, *Proc. Natl. Acad. Sci.*, 2004). Generation of mouse lines using additional female ES cell lineages is underway. We hypothesize that homoplasmic xenomitochondrial offspring must surmount a prenatal or perinatal metabolic crisis, accounting for the limited germ-line transmission results to date. We also hypothesize that these mice, when crossbred with neurodegenerative disease mouse models, will show accelerated age-related neuronal loss, due to their sub-optimal capacity for oxidative phosphorylation and putatively increased oxidative stress.



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**REGULATION OF MITOCHONDRIAL mRNA TURNOVER BY RNase-L:
INCREASED RESPIRATION AND CALCIUM UPTAKE CAPACITY IN BRAIN MITOCHONDRIA
AND CULTURED NEURONS FROM RNase-L KNOCKOUT MICE**

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Evidence indicates that inflammation contributes to ischemic and traumatic brain injury. The role of mitochondria in such inflammation-mediated injury is unknown. Exposure of cultured cells to the inflammatory cytokine, interferon, was shown to decrease the stability of mtDNA-encoded mRNA (mt-mRNA) via activation of a 2-5A-dependent RNase, called RNase-L. Increased turnover of mt-mRNA could result in reduced activity of mitochondrial gene products, resulting in impaired mitochondrial respiration and increased vulnerability to metabolic stress. In this study, we examined the role of RNase-L in regulating mitochondrial mRNA and its effect on mitochondrial bioenergetic functions. Exposure of RNase-L ^{+/+} fibroblasts to monensin, a sodium ionophore, decreased the half-life of mt-mRNA from 3h in untreated- to 1h in monensin-treated cells. In contrast, exposure of RNase-L ^{-/-} fibroblasts to monensin did not alter the half-life of mt-mRNA. Brain mitochondria prepared from RNase-L ^{-/-} mice showed higher State 3 and State 4 respiration and 40% higher calcium uptake capacity compared to mitochondria prepared from RNase-L ^{+/+} mice. Cerebellar granule neurons from RNase-L ^{-/-} mice exhibited higher calcium buffering capacity compared to RNase-L ^{+/+} neurons, after exposure to the excitotoxic amino acid glutamate. The role of RNase-L activation as a contributor of neuronal cell death via mitochondrial dysfunction will be determined by comparing the susceptibility of RNase-L ^{+/+} and RNase-L ^{-/-} mice to focal ischemic injury. Supported by AHA 0256383U, NIH NS045081, and DAMD 17-99-1-9483.

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**CALCIUM REGULATION OF BRAIN MITOCHONDRIAL CALCIUM/cAMP RESPONSE ELEMENT
BINDING PROTEIN (CREB)**

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Calcium-mediated signaling regulates nuclear gene transcription by CREB via calcium-dependent kinases and phosphatases. This study tested the hypothesis that CREB is also present in mitochondria and is subject to dynamic calcium-dependent modulation of its phosphorylation state. Antibodies to CREB and phosphorylated CREB (pCREB) were used to demonstrate the presence of both forms in highly purified mitochondrial fractions isolated from rat brain. When energized mitochondria were exposed to physiological levels of Ca²⁺, pCREB levels were reduced while total CREB levels remained constant. In the presence of the inner mitochondrial membrane uniporter inhibitor RU360, Ca²⁺-dependent loss of pCREB levels was attenuated. This suggests that intramitochondrial calcium plays an important role in pCREB dephosphorylation. Exposure of mitochondria to the pore-forming molecule alamethicin resulted in osmotic swelling, disruption of the outer membrane, and a reduction in CREB but not pCREB mitochondrial immunoreactivity. These results suggest that pCREB is located exclusively in the mitochondrial matrix or inner membrane while CREB resides in the intermembrane space. Thus, a calcium-dependent phosphatase may regulate the intramitochondrial distribution of mitochondrial pCREB/CREB and possibly the transcriptional activating activity of mitochondrial pCREB/CREB. This form of regulation may affect mitochondrial gene expression following acute brain injury as early, post-ischemic pCREB dephosphorylation and delayed pCREB hyperphosphorylation were observed in brain mitochondria isolated following global cerebral ischemia and reperfusion. (Supported by NIEHS ES07263, NIH NS34152, DAMD 17-99-1-9483 and AHA 0256359U).



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THE UNCOUPLING AGENT 2,4-DINITROPHENOL IMPROVES MITOCHONDRIAL FUNCTION AND REDUCES MEASURES OF OXIDATIVE DAMAGE IN THE INJURED SPINAL CORD

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Secondary injury following traumatic spinal cord injury (SCI) plays an important role in ongoing tissue loss. While many factors contribute to secondary injury, mitochondrial function is central to determining cell survival under a variety of pathophysiological conditions. Therefore, strategies that target mitochondrial function may prove beneficial in the treatment of secondary damage in SCI. Recent evidence suggests that mild mitochondrial uncoupling may limit cellular demise by reducing the release and/or production of damaging reactive oxygen species and other cell death signaling molecules. The potential importance of mitochondrial uncoupling in mammals is underscored by the endogenous presence of uncoupling proteins in the central nervous system. The purpose of this study was to investigate the potential neuroprotective efficacy of the mitochondrial uncoupler 2,4-dinitrophenol (DNP) in rats following a mild to moderate spinal cord contusion injury. Animals received intraperitoneal injections of vehicle (DMSO) or 5mg/ml of DNP at 15 minutes prior to injury and twenty-four hours later mitochondrial function was assessed in isolated mitochondria from spinal cord synaptosomes. In addition, synaptosomes were used to measure indicators of reactive oxygen species formation, lipid peroxidation, and protein oxidation. Relative to control, pretreatment with DNP significantly improved mitochondrial function and decreased reactive oxygen species levels, lipid peroxidation, and protein carbonyl content following spinal cord injury. Furthermore, pretreatment with DNP significantly increased the amount of remaining white matter at the injury epicenter 6 weeks after injury. These results indicate that treatment with mitochondrial uncoupling agents may provide a novel approach for the treatment of secondary injury following spinal cord contusion. Future studies examining postinjury treatment paradigms will reveal the therapeutic potential of strategies targeting mitochondrial function. Supported by PHS grants NS40015, NS046380, and the Kentucky Spinal Cord and Head Injury Research Trust (JES) and NS048191 (PGS).

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2,4-DINITROPHENOL ATTENUATES QUINOLINIC ACID- AND ISCHEMIA-INDUCED MITOCHONDRIAL DYSFUNCTION

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It is now generally accepted that excitotoxic cell death involves bioenergetic failure resulting from the uptake of Ca^{2+} and the generation of reactive oxygen species (ROS) by mitochondria. Both Ca^{2+} uptake and ROS formation by the mitochondria are dependent on the mitochondrial membrane potential ($\Delta\Psi_m$) that results primarily from the proton gradient across the inner membrane. Mitochondrial uncoupling refers to a condition in which the leak of protons back into the matrix bypasses ATP synthase. As a consequence of this "short-circuit", there is a reduction in $\Delta\Psi_m$. To determine whether uncoupling prevents mitochondrial dysfunction caused by excitotoxic insults, we have assessed the effects of the classic uncoupling agent 2,4-dinitrophenol (DNP) in two models of brain damage involving excitotoxic pathways. In the first study, rats were pretreated with DNP i.p. and one hour later injected with 90nmol/ μl QA in the right striatum. In the second study, animals underwent 2 hours of middle cerebral artery occlusion and one hour after reperfusion commenced, animals were systemically administered DNP. In both animal models, treatment with DNP 1) attenuated mitochondrial ROS formation, 2) decreased mitochondrial Ca^{2+} levels and 3) improved mitochondrial function as determined by an enhancement of oxygen consumption. We have previously shown that DNP confers ~25% protection even when administered 3 hrs after infusion of QA (*Brain Res*, 966: 312-6, 2003). In this study, tissue damage induced by ischemia/reperfusion was reduced by ~40% in DNP-treated animals compared to vehicle-treated animals. These data indicate that DNP may confer protection against acute brain injury involving excitotoxic pathways by mechanisms that improve mitochondrial homeostasis. Supported by DA13144 and NS42111 (WFM), NS47395 (LCP) and NS48191 (PGS).

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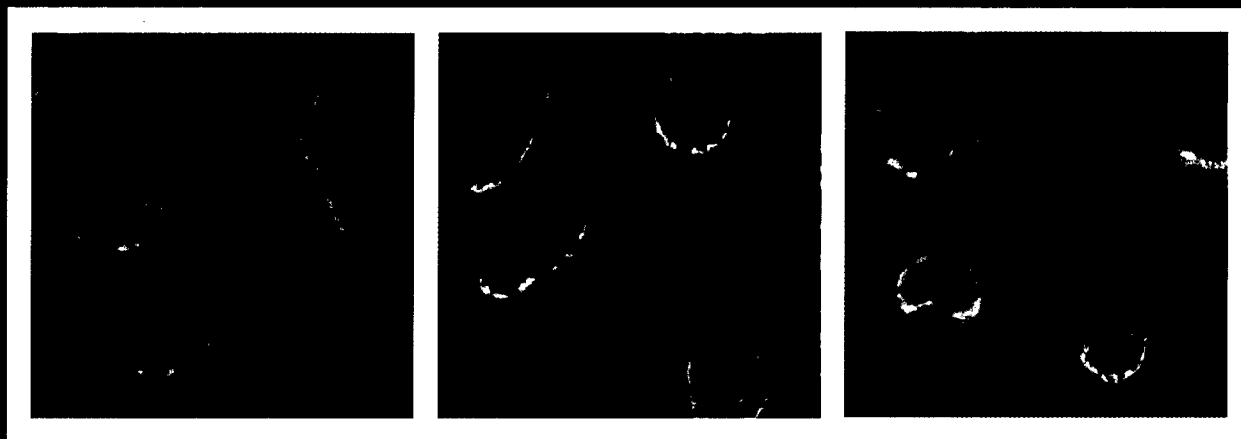
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**JOURNAL OF BIOENERGETICS AND
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Mitochondria and Neuroprotection—In Memory
of Albert L. Lehninger**

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Front Outside Cover: HT22 cells stably transfected with mitochondrially-targeted eYFP. Cells were also stained with Hoechst 33342 to label nuclei (Fig. 2, Reynolds *et al.*).

Introduction

Mitochondria and Neuroprotection—In Memory of Albert L. Lehninger

Gary Fiskum¹

This issue represents the proceedings of the Mitochondria and Neuroprotection Symposium held in Fort Lauderdale Florida on April 16–19, 2004. In addition to the minireviews authored by plenary session speakers, four research communications are included representing results presented at the poster sessions. Over 130 scientists from many different countries attended the symposium and 40 posters were presented. The symposium was held in memory of Dr Albert Lester Lehninger, one of the most highly regarded biochemists and medical educators of the twentieth century, whose contributions to the field of mitochondrial bioenergetics form the foundation of much of the research discussed in this special issue.

Albert Lehninger was born in Bridgeport, Connecticut, on February 17, 1917, and received a BA in English from Wesleyan University in 1939. He received his PhD in physiological chemistry from the University of Wisconsin in 1942. Lehninger stayed on at the University of Wisconsin as an Instructor until 1945, when he was appointed Assistant Professor of biochemistry and surgery at the University of Chicago. At this juncture he had published 11 research articles. From 1945 to 1952, he published an additional 33 articles. These studies included the discovery that fatty acid oxidation, ketone body metabolism, and the TCA cycle occur in the mitochondrion, some of the first investigations employing isolation of this organelle (see e.g., JBC 1949 and 1950). Thus, while Lehninger was a chemist at heart, he was one of the founding fathers of cell biology. In 1952 at the tender age of 33, Albert Lehninger was appointed the Chair of Physiological Chemistry at the Johns Hopkins University School of Medicine and retained that position until 1978. He developed this department into what was and still is one of the most outstanding departments of biochemistry in the world. In



Albert L. Lehninger 1917–1986.

the 10 year span of 1952–1962, he published 88 articles. These studies helped elucidate the roles of different electron transport chain complexes in energy metabolism and identified the mitochondrion as a site of action of thyroid hormone. Moreover, his research established many principles of metabolic regulation that apply to all aspects of cellular homeostasis. In the next 10 years, he published 100 articles. This era included elucidation of how mitochondrial calcium uptake was coupled to respiration. He and his coworkers also contributed to the understanding of respiratory uncoupling and mitochondrial transport of ATP and ADP during this period. In 1970, he published an article on “Comparative studies on mitochondria isolated from neuron-enriched and glia-enriched fractions of the brain,” a topic that is still very timely and closely related to the subject of this

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symposium. During the next several years, the Lehninger lab expanded on the comparative biology theme, characterizing mitochondrial heterogeneity among different normal tissues, and between normal and neoplastic cells. The 1970s was a period of intense controversy concerning the mechanism of oxidative phosphorylation. While Lehninger's research strongly supported Peter Mitchell's chemiosmotic coupling hypothesis, his meticulous measurements of the stoichiometric relationships between oxygen consumption, proton efflux, and calcium and phosphate uptake helped refine the hypothesis into a generally accepted mechanism. The last approximately 10 years of his career focused on the roles of mitochondrial enzymes and transporters in physiological and pathological energy metabolism and cell calcium homeostasis. These investigations included a comparison of the calcium uptake affinities

of mitochondria and endoplasmic reticulum, and how mitochondrial respiration is inhibited by a factor released by macrophages, later identified as nitric oxide. In total, Lehninger published 310 research articles. In addition, he was the sole author of seven books, including "The Mitochondrion" in 1964, the first edition of "Bioenergetics" in 1965, and three editions of his world-famous Biochemistry textbook, published in over 12 different languages. His numerous additional honors and activities include six honorary doctorates, election to the National Academy of Sciences, and election as President of the American Society of Biological Chemists. Al Lehninger passed away on March 6, 1986. In addition to all his academic accomplishments, he trained scores of scientists and educators from every corner of the globe, and was admired and loved by all who had the good fortune to know him.

The Integration of Mitochondrial Calcium Transport and Storage

David G. Nicholls^{1,2} and Susan Chalmers¹

Received March 15, 2004; accepted May 7, 2004

The extraordinary capacity of isolated mitochondria to accumulate Ca^{2+} has been established for more than 40 years. The distinct kinetics of the independent uptake and efflux pathways accounts for the dual functionality of the transport process to either modulate matrix free Ca^{2+} concentrations or to act as temporary stores of large amounts of Ca^{2+} in the presence of phosphate. One puzzle has been the nature of the matrix calcium phosphate complex, since matrix free Ca^{2+} seems to be buffered in the region of 1–5 μM in the presence of phosphate while millimolar Ca^{2+} remains soluble in *in vitro* media. The key seems to be the elevated matrix pH and the third-power relationship of the PO_4^{3-} concentration with pH. Taking this into account we may now finally have a model that explains the major features of physiological mitochondrial Ca^{2+} transport.

KEY WORDS: Mitochondria; calcium; phosphate; membrane potential; tricalcium phosphate; pH; hydroxyapatite.

As so ably documented by the studies of Al Lehninger and his collaborators in the 1960s and 1970s, one of the most fascinating properties of isolated mitochondria is their seemingly enormous capacity (frequently in excess of 1 micromole/mg protein—corresponding to a total matrix concentration of 1 M) to accumulate and retain calcium. In their classic studies (reviewed in Lehninger, 1974; Lehninger *et al.*, 1967; Rossi and Lehninger, 1964) some of the major features were established, including the role of phosphate as accompanying ion, effects on respiration, and the detection of associated proton movements, although this last was initially ascribed to the passive response to a primary Ca^{2+} transport process rather than a manifestation of the chemiosmotic hypothesis. With the gradual realization that this latter provided a mechanism for the uptake of Ca^{2+} via a uniport channel came a new problem, namely that estimates of the membrane potential $\Delta\psi$ in the region of 150 mV (Nicholls, 1974) and thus capable of maintaining a 10^5 gradient of free Ca^{2+} across the inner membrane led to the conclusion that a uniport would

lead to an irreversible accumulation of the cation into the matrix. This paradox was resolved by the discovery of independent efflux pathways in heart and liver mitochondria (Crompton *et al.*, 1978; Crompton and Heid, 1978) leading to the concept of a continuous cycling of Ca^{2+} across the membrane utilizing the proton gradient either directly in liver mitochondria, via the $\text{H}^+/\text{Ca}^{2+}$ exchanger, or indirectly in the case of heart or brain mitochondria where a combination of $\text{Na}^+/\text{Ca}^{2+}$ and H^+/Na^+ exchangers were operative (Fig. 1).

This cycling of Ca^{2+} led to a wealth of papers focussing on the ability of the pathways to transmit changes in cytoplasmic free Ca^{2+} , $[\text{Ca}^{2+}]_c$, into the matrix to control key metabolic enzymes including pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and NAD-linked isocitrate dehydrogenase (reviewed in Monje *et al.*, 2001). However, it was still important to establish why mitochondria accumulated Ca^{2+} rather than simply cycling it. Our own investigations began with the acquisition of a Ca^{2+} selective macroelectrode and an investigation of the extent to which mitochondria could reduce the extramitochondrial free Ca^{2+} concentration, $[\text{Ca}^{2+}]_e$ (Nicholls, 1978). Respiring liver mitochondria incubated in the presence of 30 nmol total Ca^{2+} /mg protein could lower $[\text{Ca}^{2+}]_e$ to about 0.8 μM while if $[\text{Ca}^{2+}]_e$ was lowered below this

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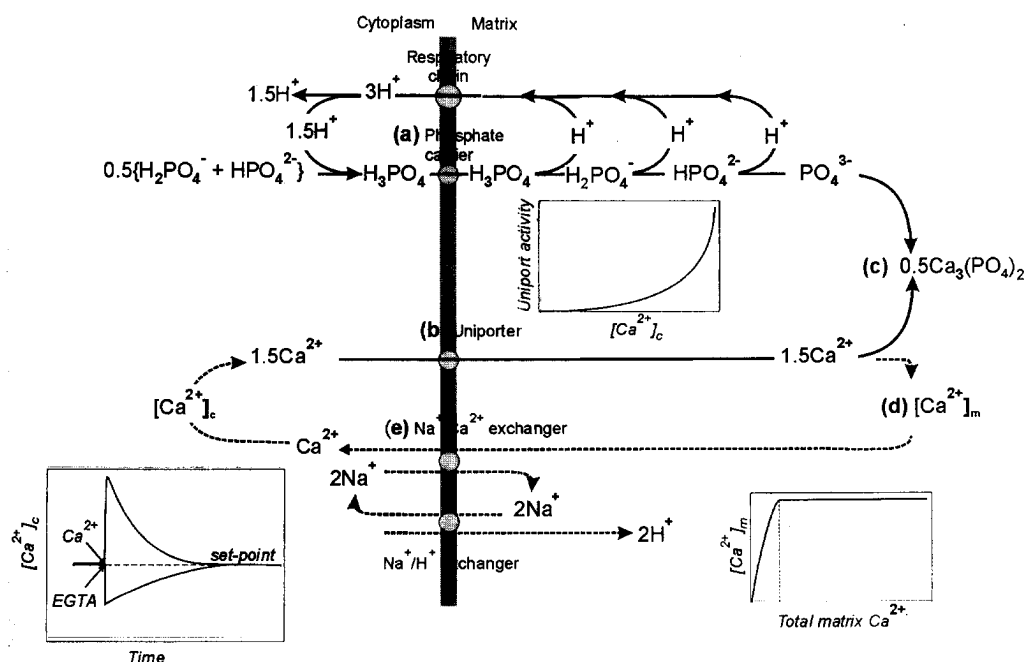


Fig. 1. Schematic of the ion movements involved in the net accumulation (solid arrows) and steady-state cycling (dashed arrows) of Ca^{2+} . (a) The phosphate carrier transports H_2PO_4^- in exchange for OH^- but this is formally equivalent to the electroneutral transport of H_3PO_4 . Because three proton dissociations are required to form PO_4^{3-} the concentration of this species is inversely proportional to the cube of the proton concentration in the matrix (Chalmers and Nicholls, 2003). (b) The uniport activity increases as the 2.5 power of cytoplasmic free Ca^{2+} concentration $[\text{Ca}^{2+}]_c$ (Zoccarato and Nicholls, 1982), see insert. (c) The tricalcium phosphate complex forms when its ion activity product is exceeded. Because the concentration of PO_4^{3-} increases with pH, the solubility of Ca^{2+} decreases and is about $2 \mu\text{M}$ when matrix pH is about 7.7 and external total phosphate is about 5 mM (Chalmers and Nicholls, 2003). (d) The matrix free Ca^{2+} concentration, $[\text{Ca}^{2+}]_m$, varies with total matrix Ca^{2+} until about 10 nmol/mg is accumulated and the tricalcium phosphate complex start to form. In this initial region matrix Ca^{2+} can regulate tricarboxylic acid enzymes. Once the complex forms, $[\text{Ca}^{2+}]_m$ is invariant with matrix Ca^{2+} load and the cytoplasmic Ca^{2+} buffering mode is seen (see insert). (e) The $\text{Na}^+/\text{Ca}^{2+}$ exchanger is controlled by $[\text{Ca}^{2+}]_m$; when the matrix is in cytoplasmic buffering mode ($>10 \text{ nmol } \text{Ca}^{2+}/\text{mg}$ accumulated) mitochondria seek to accumulate (or release matrix Ca^{2+} to restore a set-point at which the kinetics of uptake via the uniporter exactly balance efflux via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Nicholls, 1978).

value by the addition of a chelator there was a slow release of matrix Ca^{2+} until this same value was attained (Nicholls, 1978). It thus seemed that this represented a value at which a dynamic steady-state equilibrium cycling was achieved.

The equilibrium $[\text{Ca}^{2+}]_e$, which we termed the "set-point" was invariant when the total Ca^{2+} load was varied from 10 to 50 nmol/mg protein (Nicholls, 1978). However above this value the capacity of the mitochondria to accumulate Ca^{2+} was severely curtailed and the set-point rose. Net accumulation of Ca^{2+} would be predicted to lower $\Delta\psi$ and raise ΔpH as net proton extrusion occurs in compensation for the uptake of Ca^{2+} . The only reason why 50 nmol/mg protein of Ca^{2+} could be accumulated without affecting the set-point was because of the presence of endogenous phosphate in the preparation. When this was depleted by preincubating mitochondria with glucose and

hexokinase or by inhibiting the phosphate transporter with *N*-ethylmaleimide much less Ca^{2+} could be taken up before the set-point was raised (Nicholls, 1978; Zoccarato and Nicholls, 1982). In theory, both acetate and phosphate could serve as compensatory permeant anions to prevent the build-up of ΔpH and drop in $\Delta\psi$; however their effects were distinct. With acetate the set-point rose slowly with Ca^{2+} load (Nicholls, 1978; Zoccarato and Nicholls, 1982) whereas with additional phosphate as permeant anion the set-point remained remarkably constant as total Ca^{2+} was varied from 10 to several hundred nmol/mg (Nicholls and Scott, 1980). As investigated by Rossi and Lehninger (1964) matrix Ca^{2+} in the presence of phosphate appears to form some type of osmotically inactive complex, whereas calcium acetate is soluble. This raised the possibility that the remarkable invariance of the set-point over the range from 10 to 500 nmol/mg Ca^{2+} (Chalmers and

Nicholls, 2003) was due to the kinetic balance between the activity of the uniporter at a given $[Ca^{2+}]_e$ and an activity of the efflux pathway that was essentially independent of total matrix Ca^{2+} load over this range because the free matrix Ca^{2+} , $[Ca^{2+}]_m$, was buffered at a constant value by the supposed Ca^{2+} -phosphate complex.

This hypothesis was tested in a subsequent study (Zoccarato and Nicholls, 1982). Without making any assumptions about the chemical nature of the Ca^{2+} -phosphate complex, a constant ion activity ("solubility") product should mean that $[Ca^{2+}]_m$ would decrease when the matrix free phosphate concentration increased. This latter is surprisingly easy to control. Because the dominant pathway for Pi transport across the inner membrane is the electroneutral phosphate transporter (Palmieri *et al.*, 1996), then if ΔpH does not change the concentrations of the different ionized forms of Pi free in the matrix will be proportional to the external Pi concentration. This was tested directly by monitoring the net rate of Ca^{2+} efflux from liver mitochondria following addition of ruthenium red to inhibit the uniporter. Ca^{2+} efflux rates decreased from 7 nmol/min/mg protein from mitochondria phosphate depleted by glucose/hexokinase to 0.5 nmol/min/mg protein in the presence of 3 mM external Pi (Zoccarato and Nicholls, 1982). Mitochondrial membrane potential did not change when Pi was increased, but the set-point decreased from 0.78 μM to 0.55 μM .

The rather modest change in set-point accompanying a 16-fold decrease in efflux rate indicates a very steep dependency of the uniporter on $[Ca^{2+}]_e$. This was investigated directly by an experiment in which Ca^{2+} was infused into mitochondrial incubations at varying rates and $[Ca^{2+}]_e$ was monitored until it achieved a constant value, implying that the net rate of Ca^{2+} accumulation by the mitochondria equalled the rate of the infusion and hence that the activity of the uniporter was equal to the infusion rate plus the activity of the efflux pathway. By varying the infusion rate the activity of the uniporter was determined to vary as the 2.5th power of $[Ca^{2+}]_e$. The contrast between this high dependency for uptake and the virtual independency of the efflux pathway on matrix Ca^{2+} load (due to the buffering of $[Ca^{2+}]_m$) is sufficient to account for the remarkable constancy of the set-point over this 50-fold range of total matrix Ca^{2+} from 10 to 500 nmol/mg.

It should be emphasized in these studies that care was taken to reproduce the physiological conditions existing in the cytoplasm as accurately as possible, and in particular including adenine nucleotides in the incubation media. As evidenced from the literally thousands of publications on the permeability transition (PT), it is all too easy to incubate mitochondria in sucrose under nonphysiological conditions, omit the natural protective action of exogenous

adenine nucleotides, subject the unfortunate organelles to a massive bolus of Ca^{2+} and watch them swell. This is not to denigrate carefully controlled experiments designed to reproduce oxidative stress in, for example cardiac reperfusion models (Crompton, 1999), but rather to emphasize the importance of realizing that mitochondria operate in a controlled environment.

We have recently revisited the question of the nature of the matrix stored Ca^{2+} (Chalmers and Nicholls, 2003) in order to answer a number of outstanding questions. In particular we were interested in confirming that the Ca^{2+} -phosphate complex in the matrix was able to maintain $[Ca^{2+}]_m$ at a constant value independent of total Ca^{2+} load and attempting to determine the factors that control the maximal Ca^{2+} loading capacity of the matrix. We were concerned to eliminate the bioenergetic loads associated with conventional bolus additions of the cation which result in a sudden demand on the proton gradient, transient depolarization, increased respiration, and changes in ΔpH . Because each of these parameters changes rapidly during a bolus addition it is difficult to distinguish the precise factors that are for example responsible for defining the maximal capacity of the matrix to retain Ca^{2+} . Instead, we adapted the infusion technique discussed above in order to slowly load the matrix with Ca^{2+} with negligible consequences for the mitochondrial bioenergetics.

Liver mitochondria in the presence of ADP and oligomycin were able to accumulate 800 nmol Ca^{2+} /mg over a period of 10 min before $[Ca^{2+}]_e$ rose precipitously indicating the onset of a PT. No change in mitochondrial membrane potential occurred during the infusion until the onset of the transition. Some increase in NAD(P)H fluorescence was noted, together with an increase in light-scattering. It should be emphasized in this context that what is usually referred to as "swelling" is actually measured as a decrease in light scattering caused by a decrease in the difference in refractive index between the matrix and the medium as the latter enters and dilutes the former. The increased light scattering during matrix Ca^{2+} loading is most likely due to the light-scattering properties or increased matrix refractive index due to the formation of the matrix Ca^{2+} -phosphate complex. Importantly, during the Ca^{2+} loading no increase in the level of reactive oxygen species could be detected, if anything H_2O_2 production decreased during Ca^{2+} loading (Chalmers and Nicholls, 2003).

The PT inhibitor cyclosporin A enhances the Ca^{2+} loading capacity of liver mitochondria in the presence of ADP by 2.5-fold, while oxidation of endogenous NADH by the addition of acetoacetate decreases the loading capacity by a similar extent (Chalmers and Nicholls, 2003). Interestingly acetoacetate is still effective in lowering

loading capacity in the presence of cyclosporin A and ADP.

The nature of the Ca^{2+} -phosphate complex and its ability to buffer $[\text{Ca}^{2+}]_m$ that was predicted by the earlier studies on the set-point were investigated by loading rat brain mitochondria with the low affinity fura2-FF. As the total matrix load was varied either by the addition of Ca^{2+} or EGTA it became apparent that there was a discontinuity at about 10 nmol total Ca^{2+} /mg protein. Above this value $[\text{Ca}^{2+}]_m$ was virtually invariant with total load, whereas below 10 nmol/mg $[\text{Ca}^{2+}]_m$ varied as a linear function of total Ca^{2+} . This resolves a longstanding debate between advocates of matrix Ca^{2+} as a means of transmitting hormonal and metabolic changes in cytoplasmic Ca^{2+} into the matrix (Hansford, 1994; McCormack and Denton, 1990) and those including ourselves who had emphasized the Ca^{2+} buffering function of the mitochondrion (Nicholls and Åkerman, 1982). It is apparent that the mitochondrion is beautifully adapted to either role. When $[\text{Ca}^{2+}]_c$ is maintained below the set-point there is too little matrix Ca^{2+} to form the Ca^{2+} -phosphate complex and $[\text{Ca}^{2+}]_m$ varies with $[\text{Ca}^{2+}]_c$, allowing for a messenger role for the cation and the control of the citric acid cycle. When however $[\text{Ca}^{2+}]_c$ rises, even briefly, above the set-point then sufficient matrix loading occurs for the formation of the Ca^{2+} -phosphate complex.

Studies with fluorescent Ca^{2+} indicators using either isolated mitochondria (Al Nassar and Crompton, 1986; Davis *et al.*, 1987; Lukacs and Kapus, 1987; McCormack *et al.*, 1989; Moreno and Hansford, 1988) or intact cells (numerous studies employing rhod-2 and related indicators) have reported surprisingly low values for $[\text{Ca}^{2+}]_m$ in the range 1–5 μM under a variety of conditions. Indeed in the regulatory range (0–10 nmol total Ca^{2+} /mg) the effects on citric acid cycle enzymes are consistent with $[\text{Ca}^{2+}]_m$ values of 0.5–2 μM (Hansford and Castro, 1982). In our study $[\text{Ca}^{2+}]_m$ for brain mitochondria remained in the range 2–3 μM when total Ca^{2+} was increased from 10 to 500 nmol/mg (Chalmers and Nicholls, 2003). Thus the earlier prediction that $[\text{Ca}^{2+}]_m$ should be essentially independent of total matrix Ca^{2+} (Zoccarato and Nicholls, 1982) was confirmed. Furthermore, the expected inverse relationship between external P_i concentration and $[\text{Ca}^{2+}]_m$ was also found. Interestingly cyclosporin A, although it more than doubled the maximal loading capacity of the matrix did not affect the stability of the Ca^{2+} -phosphate complex.

A major puzzle has been how to reconcile the apparent properties of the matrix Ca^{2+} -phosphate complex with that of known complexes in solution. Physiological cell incubation media contain millimolar Ca^{2+} in solution in the presence of millimolar P_i , and yet in the matrix some form

of osmotically inactive complex forms when $[\text{Ca}^{2+}]_m$ rises above 1–5 μM . Furthermore, in vitro calcium phosphate complexes once formed are notoriously difficult to redissolve, whereas the addition of a protonophore to Ca^{2+} -loaded mitochondria leads to an extremely rapid efflux of Ca^{2+} via reversal of the uniporter and phosphate separately via the phosphate transporter (Zoccarato and Nicholls, 1982). Finally, if the gradient of free Ca^{2+} across the inner membrane is really so low (say 0.5 μM outside and 2 μM inside), as is the gradient of free P_i (defined by the low ΔpH) why is the addition of protonophore so effective in triggering massive and rapid Ca^{2+} release?

It is not possible to investigate the nature of the matrix Ca^{2+} -phosphate complex directly, due to its instant dissociation when the mitochondria are disrupted and to the ability of Ca^{2+} -phosphate complexes to change during fixation or drying for physicochemical analysis. Thus hydroxyapatite can be detected in fixed and desiccated samples, but it is generally accepted that this is an artifact. In an artificial cytoplasm in the presence of ATP, amorphous $\text{Ca}_3(\text{PO}_4)_2$ is initially formed when millimolar Ca^{2+} is titrated in, particularly at alkaline pH (Wuthier *et al.*, 1985).

Some limits on the stoichiometry of the matrix complex can be reached by accurate determination of the ratio of Ca^{2+} accumulated to net protons extruded during the uptake. These classic studies by Lehninger and colleagues produced values close to 1.0 $\text{H}^+/\text{Ca}^{2+}$ (Lehninger *et al.*, 1967). While it was not initially realized that this was a "chemiosotic" proton extruded by the respiratory chain the analysis remains valid. This ratio is consistent with the formation of $\text{Ca}_3(\text{PO}_4)_2$ (Fig. 1) whereas hydroxyapatite with a formula $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ would give a ratio of 1.1. While these ratios are perhaps too close to allow unambiguous discrimination, other forms such as CaHPO_4 (ratio 0.5) and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (ratio-1) can be eliminated.

The clue both to the low Ca^{2+} solubility in the matrix and the rapid efflux initiated by protonophore appears to lie in the pH gradient across the inner membrane. The highly active phosphate carrier equilibrates the transported species H_2PO_4^- with OH^- and thus accumulates the anion as a function of the ΔpH . Two further dissociations of H_2PO_4^- to HPO_4^{2-} and PO_4^{3-} are required before $\text{Ca}_3(\text{PO}_4)_2$ is formed and both of these dissociations are dependent on the matrix pH, with the final result that the concentration of the PO_4^{3-} species is dependent on the third power of the ΔpH at constant external phosphate.

The solubility of a salt is governed by the ion activity product (solubility product). Values reported in the literature for the calcium phosphate complexes are somewhat variable, but values of 3×10^{-30} for amorphous $\text{Ca}_3(\text{PO}_4)_2$ and 1×10^{-59} for hydroxyapatite are representative. From

the pKs for the dissociation of the phosphate anionic forms it can be calculated that the transmembrane gradient of the PO_4^{3-} anion varies as the third power of the pH gradient, so that an increase in matrix pH from 7 to 8 would increase the matrix PO_4^{3-} concentration by 1000-fold. Since the ion activity product is a constant, this means that taking an example of mitochondria incubated in the presence of 5 mM total external Pi the saturating Ca^{2+} concentration in equilibrium with tricalcium phosphate in the matrix would decrease from about 100 μM at pH 7 to 1 μM at pH 8, while the corresponding saturating Ca^{2+} concentrations in equilibrium with hydroxyapatite would be about 15-fold lower (Chalmers and Nicholls, 2003).

The best fit with the experimental data for $[\text{Ca}^{2+}]_m$ would be consistent with tricalcium phosphate in the matrix of respiring mitochondria at a pH of about 7.7, when saturating $[\text{Ca}^{2+}]_m$ would be calculated to be in the region of 2 μM . Thus, the low values reported for $[\text{Ca}^{2+}]_m$ are entirely consistent with the physical chemistry of calcium phosphate complexes at alkaline pH. This also provides an explanation for the rapid efflux of Ca^{2+} and Pi on their respective carriers when protonophores are added, since this is associated with a dramatic matrix acidification. This would decrease the concentration of PO_4^{3-} , destabilizing the complex, and increasing $[\text{Ca}^{2+}]_m$ to about 100 μM , the high Ca^{2+} gradient thus driving the rapid efflux of the cation.

The Ca^{2+} -transport properties of isolated mitochondria are sufficient to account for the major features observed for in situ mitochondrial Ca^{2+} transport. In particular mitochondria only appear to sequester significant amounts of Ca^{2+} when $[\text{Ca}^{2+}]_m$ rises above 0.5 μM , corresponding rather nicely with the set-point observed for isolated mitochondria (Werth and Thayer, 1994). Isolated mitochondria release Ca^{2+} when the external Ca^{2+} concentration falls below the set-point (Nicholls, 1978) and this behavior can be observed in cultured neurons recovering after a transient cytoplasmic Ca^{2+} load, where the recovery to basal $[\text{Ca}^{2+}]_c$ is delayed by a shoulder consistent with an unloading of the temporarily accumulated cation to the cytoplasm (Werth and Thayer, 1994).

In conclusion, the mitochondrial Ca^{2+} transport and sequestration properties are perfectly adapted to accumu-

late, store, and release large amounts of the cation. The pioneering work of Al Lehninger and his colleagues laid the essential groundwork from which these studies have evolved.

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Mitochondrial Trafficking in Neurons: A Key Variable in Neurodegeneration?

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Mitochondria are the proximate target of a number of different neurotoxins. Typically, impairing of the key bioenergetic function of mitochondria by toxins is considered as the main mechanism of action. However, the effective maintenance of energy generation in neurons depends on the biogenesis, trafficking, and degradation of mitochondria in addition to the traditional bioenergetic functions. We have recently demonstrated that glutamate alters both the trafficking and morphology of mitochondria in primary neurons. In addition, several other potential neurotoxins, including nitric oxide and zinc, inhibit mitochondrial movement and, in some cases, alter morphology too. This suggests that some part of the action of neurotoxins might include the impairment of mitochondrial trafficking in neurons, with the resultant failure of local ATP delivery.

KEY WORDS: Mitochondrial movement; excitotoxicity; zinc; fluorescence imaging; nitric oxide.

INTRODUCTION

Mitochondria have emerged as key targets of a number of different types of neurotoxin. In excitotoxic neuronal injury, activation of *N*-methyl-D-aspartate (NMDA) receptors activates a massive calcium entry that is initially buffered by mitochondria (Budd and Nicholls, 1996; White and Reynolds, 1995). However, this mitochondrial calcium loading disrupts mitochondrial function by depolarizing the mitochondrial membrane potential ($\Delta\Psi_m$) (Khodorov *et al.*, 1996; Schinder *et al.*, 1996; White and Reynolds, 1996), stimulating oxidant production (Dugan *et al.*, 1995; Reynolds and Hastings, 1995), and causing cytochrome *c* release (Budd *et al.*, 2000). Which of these events, if any, kill neurons in excitotoxicity is unclear, but preventing mitochondrial calcium accumulation effectively protects neurons from injury (Budd *et al.*, 2000; Stout *et al.*, 1998). Mitochondria are also the target of a number of neurotoxins that work more slowly than NMDA-mediated excitotoxicity. Toxins that target complex I of the electron transport chain, such as MPP⁺ and

rotenone, cause injury closely resembling Parkinson's disease in rodent models (Betarbet *et al.*, 2000; Singer *et al.*, 1987). Systemic administration of 3-nitropropionic acid, a complex II poison, selectively kills neurons in the striatum, and produces a syndrome like Huntington's disease (Beal *et al.*, 1993). Again, the critical impairment responsible for committing neurons to die following exposure to these toxins is not clear. However, a combination of energetic impairment and enhanced production of oxidants may well account for the injury.

In attempting to link mitochondrial failure to neuronal injury, the range of mitochondrial dynamics to consider in neurons extends well beyond simply ATP and oxidant production. In all cells, effective mitochondrial function depends on an intact mitochondrial life history, from biogenesis through delivery of mitochondria to appropriate cellular targets and ultimately the correct retrieval and degradation of the organelles at the end of their effective lifetime. Although the details of mitochondrial life history in neurons have not been clearly established, it is easy to appreciate the additional challenges faced by neurons because of the distances over which mitochondria must travel in order to supply ATP to regions of the cell that have high energy demands. An additional variable is that of mitochondrial morphology. It has been appreciated for some time that the shape of mitochondria varies

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considerably between cell types. Intriguingly, recent studies have suggested that morphology is a key variable of mitochondria within cells, and one that may govern the outcome from injury (Frank *et al.*, 2001; Karbowski and Youle, 2003). Here we will review recent data that show that both movement and morphology of mitochondria are affected by neurotoxins.

NEURONAL INJURY CHANGES MITOCHONDRIAL MORPHOLOGY

Several types of morphological change in mitochondria can be anticipated on the basis of current knowledge. Perhaps the most obvious is the swelling that should be associated with the induction of permeability transition, an event associated with excitotoxic neuronal injury (Reynolds and Hastings, 2001; Schinder *et al.*, 1996). In addition, the regulation of mitochondrial fission and fusion can result in a net alteration in mitochondrial shape,

and it has been proposed that fission of mitochondria is associated with apoptotic cell injury (Frank *et al.*, 2001). We recently described effects of glutamate on the morphology and movement in neurons in culture (Rintoul *et al.*, 2003). Acute application of glutamate and the subsequent influx of calcium through NMDA receptors results in a cessation of movement (discussed later), a profound alteration in morphology and also an occasional fragmentation of mitochondria (Fig. 1) that occurs within minutes of glutamate stimulation. The change in morphology is calcium dependent, and is associated with a disruption of the cytoskeleton. This morphological change could be attributed to mitochondrial swelling. However, the lack of sensitivity to cyclosporin A makes it difficult to conclude that permeability transition is the cause of the swelling. Interestingly, the glutamate-induced morphological change recovers within 1–2 h, well before neurons die from this stimulus.

Zinc is an important endogenous neurotoxin that is mobilized during ischemic brain injury (Koh *et al.*, 1997).



Fig. 1. Glutamate-induced changes in mitochondrial morphology. These images were obtained from a neuron in primary culture. The neuron expressed mitochondrially targeted enhanced yellow fluorescent protein. Panel A shows mitochondria before, while panel B was taken after, a 10-min exposure to 30 μ M glutamate. Panels C and D show the same effect with enhancement of the image to illustrate that fragmentation occurs in addition to the swelling of the mitochondria.

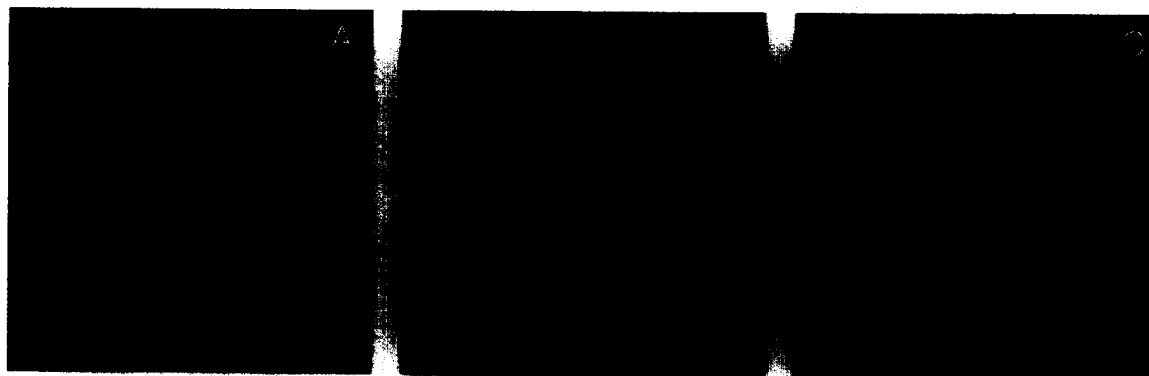


Fig. 2. Zinc alters mitochondrial morphology. This experiment was performed in HT22 cells that were stably transfected with mt-eYFP (green fluorescence) and also stained with Hoechst 33342 (red fluorescence) to label the nucleus. Panel A shows control cells, while the cells in B and C were exposed to 3 μ M zinc in the presence of 20 μ M pyrithione. Panel B was imaged 1 h after zinc exposure, while panel C was obtained after 2 h. Note the loss of filamentous structure of mitochondria as a consequence of zinc treatment.

We have recently found that zinc produces a different form of morphological change in mitochondria. We exposed HT22 cells that express mitochondrially targeted enhanced yellow fluorescent protein (mt-eYFP) to zinc in the presence of an ionophore, pyrithione. Over the next 2 h, zinc exposure resulted in nuclear condensation and a progressive fragmentation of mitochondria that precedes loss of cytochrome c (Fig. 2). This indicates that cytochrome c release is not simply a consequence of the mitochondria being shredded during the execution of apoptosis. The molecular basis for the fragmentation is not clear, but it has been shown that cells can be protected from apoptosis with a dominant negative form of the fission-promoting protein DRP1 (Frank *et al.*, 2001), and it is tempting to speculate that this response reflects an induced imbalance between mitochondrial fission and fusion that makes an important contribution to the fate of the cell.

NEURONAL INJURY CHANGES MITOCHONDRIAL MOVEMENT

As already noted, neuronal mitochondria may have to travel considerable distances to reach the site of ATP demand. Processes that disrupt the delivery of mitochondria may negatively impact neuronal viability by essentially imposing a local starvation because of the absence of necessary mitochondria. We have recently found a number of conditions that impair mitochondrial movement. The first of these was NMDA receptor activation with glutamate. As described above, glutamate alters mitochondrial morphology, but also produces a profound inhibition of mitochondrial movement (Rintoul *et al.*, 2003). In an effort to determine potential mechanisms for this effect, we

compared the actions of glutamate to the uncoupler FCCP, which depolarizes $\Delta\Psi_m$, and oligomycin, which inhibits ATP synthesis. Both of these agents inhibited movement, but did not alter morphology. This suggests that movement critically depends on ATP production by mitochondria, and further implies that the morphological change is a consequence of the calcium entry produced by glutamate but not FCCP or oligomycin (Fig. 3).

The latter conclusion suggested an additional series of experiments. It has been well established that nitric oxide is an effective inhibitor of complex IV of the electron transport chain at relatively low concentrations (Brorson *et al.*, 1999; Brown, 1999). We speculated that NO would impair mitochondrial movement. Exposing neurons to the NO donor PAPA nonoate confirmed this to be the case, and NO appears to decrease movement without altering mitochondrial morphology. We have found that several other neurotoxins decrease mitochondrial movement in neurons, including elevated intracellular zinc and oxidant exposure, but the mechanisms by which these effects occur are still under investigation.

CONCLUSIONS

These studies illustrate the point that neurotoxins can acutely alter mitochondrial movement and morphology in addition to the known effects on ATP synthesis and oxidant generation. It seems likely that inhibiting movement will result in the impairment of the delivery of mitochondria to relevant sites within neurons, although it remains to be established whether there is a specific link between the cessation of movement and injury to neurons. It should also be noted that nerve growth factor inhibits mitochondrial

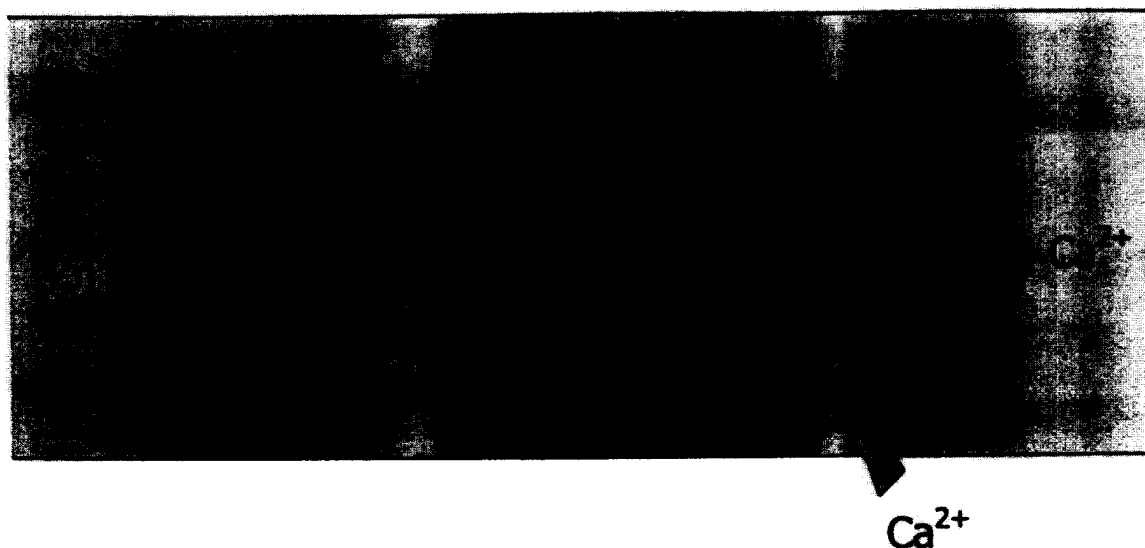


Fig. 3. Mechanisms for altering mitochondrial movement in neurons. There are likely to be three or more mechanisms for altering trafficking. Calcium can act by disrupting cytoskeletal structures like microtubules. Agents like NO and calcium can also impair movement by inhibiting ATP synthesis by mitochondria. Other neurotoxins, including zinc and some oxidizing agents inhibit mitochondrial movement without obviously depolarizing mitochondria, so this is likely to be the result of a separate mechanism.

movement in peripheral neurons (Chada and Hollenbeck, 2003). Nerve growth factor would not be considered neurotoxic in these cells, and the transient docking of mitochondria proposed as an action of the trophic factor might be considered to be a beneficial effect. It is more difficult to discern the consequence of morphological alterations for the function of neuronal mitochondria. Gross swelling as a consequence of calcium overload is probably a reflection of mitochondrial impairment, rather than a specific indicator of altered function. The more subtle fragmentation presumably yields mitochondria that are functionally intact, so that the specific hazard resulting from the decrease in mitochondrial size remains to be elucidated. However, it does raise the interesting possibility that the mechanisms that control fission or fusion might represent a novel target for neuroprotection.

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Deadly Conversations: Nuclear-Mitochondrial Cross-Talk

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Neuronal damage following stroke or neurodegenerative diseases is thought to stem in part from overexcitation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate. NMDA receptors triggered neurotoxicity is mediated in large part by activation of neuronal nitric oxide synthase (nNOS) and production of nitric oxide (NO). Simultaneous production of superoxide anion in mitochondria provides a permissive environment for the formation of peroxynitrite (ONOO⁻). Peroxynitrite damages DNA leading to strand breaks and activation of poly(ADP-ribose) polymerase-1 (PARP-1). This signal cascade plays a key role in NMDA excitotoxicity, and experimental models of stroke and Parkinson's disease. The mechanisms of PARP-1-mediated neuronal death are just being revealed. While decrements in ATP and NAD are readily observed following PARP activation, it is not yet clear whether loss of ATP and NAD contribute to the neuronal death cascade or are simply a biochemical marker for PARP-1 activation. Apoptosis-inducing factor (AIF) is normally localized to mitochondria but following PARP-1 activation, AIF translocates to the nucleus triggering chromatin condensation, DNA fragmentation and nuclear shrinkage. Additionally, phosphatidylserine is exposed and at a later time point cytochrome *c* is released and caspase-3 is activated. In the setting of excitotoxic neuronal death, AIF toxicity is caspase independent. These observations are consistent with reports of biochemical features of apoptosis in neuronal injury models but modest to no protection by caspase inhibitors. It is likely that AIF is the effector of the morphologic and biochemical events and is the commitment point to neuronal cell death, events that occur prior to caspase activation, thus accounting for the limited effects of caspase inhibitors. There exists significant cross talk between the nucleus and mitochondria, ultimately resulting in neuronal cell death. In exploiting this pathway for the development of new therapeutics, it will be important to block AIF translocation from the mitochondria to the nucleus without impairing important physiological functions of AIF in the mitochondria.

KEY WORDS: Ischemia; excitotoxicity; neurodegeneration; NMDA, nitric oxide; peroxynitrite; poly(ADP-ribose) polymerase; PARP-1.

INTRODUCTION

The extent and cost of neurologic disease is staggering. Fifty million Americans have a permanent, neuro-

logical disability that limits their daily activities. Chronic neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Lou Gehrig's disease (ALS) afflict over 6.5 million Americans. Every 53 s someone in the United States suffers a stroke, affecting over 3 million Americans each year with over 4.4 million stroke survivors who have significant disability. Clearly new strategies need to be developed to treat these patients. Basic fundamental research in cell signaling and biochemistry have begun to identify the key elements of the brain and nervous system that mediate neuronal injury. Identifying these signaling molecules would open the door to new clinical opportunities and have the potential to impact millions of lives.

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NEUROTOXICITY

Many pathways have been proposed for neuronal damage in stroke and neurodegenerative diseases including extrinsic and intrinsic apoptotic programs and excitotoxicity. Glutamate excitotoxicity is a common finding that is mediated by intracellular calcium, nitric oxide, and free radicals (Dirnagl *et al.*, 1999; Kristian and Siesjö, 1998; Lipton, 1999). Glutamate initiates its actions postsynaptically by binding to four major types of receptors: metabotropic receptors, *N*-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, and kainate receptors (Mayer and Westbrook, 1987). NMDA receptor activation mediates, in large part, glutamate excitotoxicity and neuronal damage. Glutamate-stimulated NMDA receptors flux calcium and activate a variety of intracellular calcium-dependent enzymes and processes, of which activation of neuronal NO synthase (nNOS) plays a prominent role (Samdani *et al.*, 1997). Thus, overproduction of NO from excessive or inappropriate stimulation of nNOS appears to mediate a major component of excitotoxic damage although other reactive oxygen species are also generated in excitotoxic conditions.

REACTIVE OXYGEN SPECIES (ROS)

The inherent biochemical and physiological characteristics of the brain, including high lipid concentrations and energy requirements, make it particularly susceptible to free-radical-mediated insult. When oxygen-free radicals are generated in excess of a cell's antioxidant capacity severe damage to cellular constituents including proteins, DNA, and lipids (Chan, 2001) can occur. The oxygen species that are typically linked to oxidative stress are superoxide anion, hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite (ONOO^-). In mitochondria generation of these species from molecular oxygen is a normal aspect of mammalian respiration, and over activation of the NMDA receptor results in the increased formation of reactive oxygen species (Chan, 2001). NMDA-receptor-mediated stimulation of phospholipase A_2 and the subsequent release of arachidonic acid, prostaglandins, leukotrienes, thromboxanes, and platelet-activating factor leads to a variety of toxic events including generation of oxygen-free radicals (Dirnagl *et al.*, 1999). These processes can cause the neuron to digest itself by protein breakdown, free-radical formation, and lipid peroxidation. Under conditions of calcium elevation and energy failure, xanthine dehydrogenase is converted to xanthine oxidase, the activity of

which results in superoxide anion formation (Chan, 2001). The superoxide anion is generated by multiple pathways and is often placed at the start of an oxidative stress cascade. The brain derives most of its energy exclusively from oxidative respiration through the mitochondrial electron transport chain. Mitochondria are located throughout the neuronal perikarya and its processes. During the production of ATP there is a small high-energy electron "leak" (1–3%) resulting in the generation of superoxide anion. Superoxide anion is constrained by membranes that it cannot cross and is retained within mitochondria. Superoxide dismutase (SOD) catalyzes the conversion of superoxide anion to H_2O_2 , which is diffusible within and between cells (Chan, 2001). NO is synthesized on demand by the enzyme NOS from the essential amino acid, L-arginine (Dawson and Dawson, 1998). There are three NOS genes, neuronal NOS (nNOS), endothelial NOS, and immunologic NOS that were named by the tissue from which they were first cloned. NO is small, diffusible, membrane permeable and reactive. The biochemical reactions involving NO are not well characterized. Probably the most important oxidant involved in the genesis of neurotoxicity is peroxynitrite (ONOO^-). Peroxynitrite is formed from the reaction of NO with superoxide anion. In vitro the rate of this reaction is three times faster than the rate of reaction of the enzyme, SOD, in catalyzing the dismutation of superoxide anion (Ischiropoulos and Beckman, 2003). Therefore, when present at appropriate concentrations, NO can effectively compete with SOD for superoxide anion. Although a simple molecule, peroxynitrite is chemically complex. It has the activity of hydroxyl radical and nitrogen dioxide radical, although it does not readily decompose into these entities. Peroxynitrite can also directly nitrate and hydroxylate aromatic rings on amino acid residues. It is also a potent oxidant that reacts readily with sulfhydryls, with zinc-thiolate, lipids, proteins, and DNA (Ischiropoulos and Beckman, 2003).

POLY(ADP-RIBOSE) POLYMERASE (PARP)

The discovery that inhibitors of poly (ADP-ribose) polymerase (PARP) are neuroprotective against NMDA and NO neurotoxicity initiated interest in PARP activity in the CNS (Eliasson *et al.*, 1997; Endres *et al.*, 1997; Zhang *et al.*, 1994). PARP-1 is a member of a growing family of proteins that now includes 18 putative PARP proteins based on protein domain homology and enzymatic function. PARP-1 is the PARP responsible for large branch chain polymers and generates >95% of the poly(ADP-ribose) in a cell. The obligatory trigger of PARP-1 activation is DNA strand nicks and breaks, which can be induced by a

variety of environmental stimuli and free radical/oxidant attacks, including oxidants (hydrogen peroxide, hydroxyl radical, peroxynitrite), ionizing radiation, and genotoxic agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). In response to DNA damage, PARP-1 becomes activated and, using NAD^+ as a substrate, it builds up polymers of adenosine diphosphate ribose (de Murcia and Menissier de Murcia, 1994; Lindahl *et al.*, 1995). Poly(ADP-ribose) acceptors include histones, topoisomerases I and II, DNA polymerases, and DNA ligase 2, as well as PARP-1 itself. Poly-ADP-ribosylation might result in an inhibition of the activity of some of these enzymes. In the case of histones, poly-ADP-ribosylation stimulates chromatin relaxation. The physiological function of PARP-1 and poly (ADP-ribosylation) is still under heavy debate. From studies using pharmacological inhibitors of PARP-1, poly(ADP-ribosylation) has been suggested to regulate gene expression and gene amplification, cellular differentiation and malignant transformation, cellular division, and DNA replication, as well as apoptotic cell death (Chiarugi, 2002).

Pharmacologic inhibition of PARP-1 or genetic knockout of PARP-1 elicits cytoprotection in a variety of disease models including ischemia-reperfusion injury, diabetes, inflammatory-mediated injury, reactive oxygen species-induced injury, glutamate excitotoxicity, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injury (Eliasson *et al.*, 1997; Endres *et al.*, 1997; Mandir *et al.* 1999; Szabo and Dawson, 1998; Yu *et al.*, 2002; Zhang *et al.* 1994). How PARP-1 activation kills neurons is not known. A cell suicide hypothesis was developed in the 1980s (Berger and Berger, 1986; Berger *et al.*, 1983) and has been used to explain the action of PARP-1 in the CNS (Fig. 1). The suicide theory is based on the role of cellular NAD^+ to regulate an array of vital cellular processes. NAD^+ serves as a cofactor for glycolysis and the tricarboxylic acid cycle, thus providing ATP for most cellular processes (Hageman and Stierum, 2001). NAD^+ also serves as the precursor for NADP, which acts as a cofactor for the pentose shunt, for bioreductive synthetic pathways, and is involved in the maintenance of reduced glutathione pools (Hageman and Stierum, 2001). The observation that

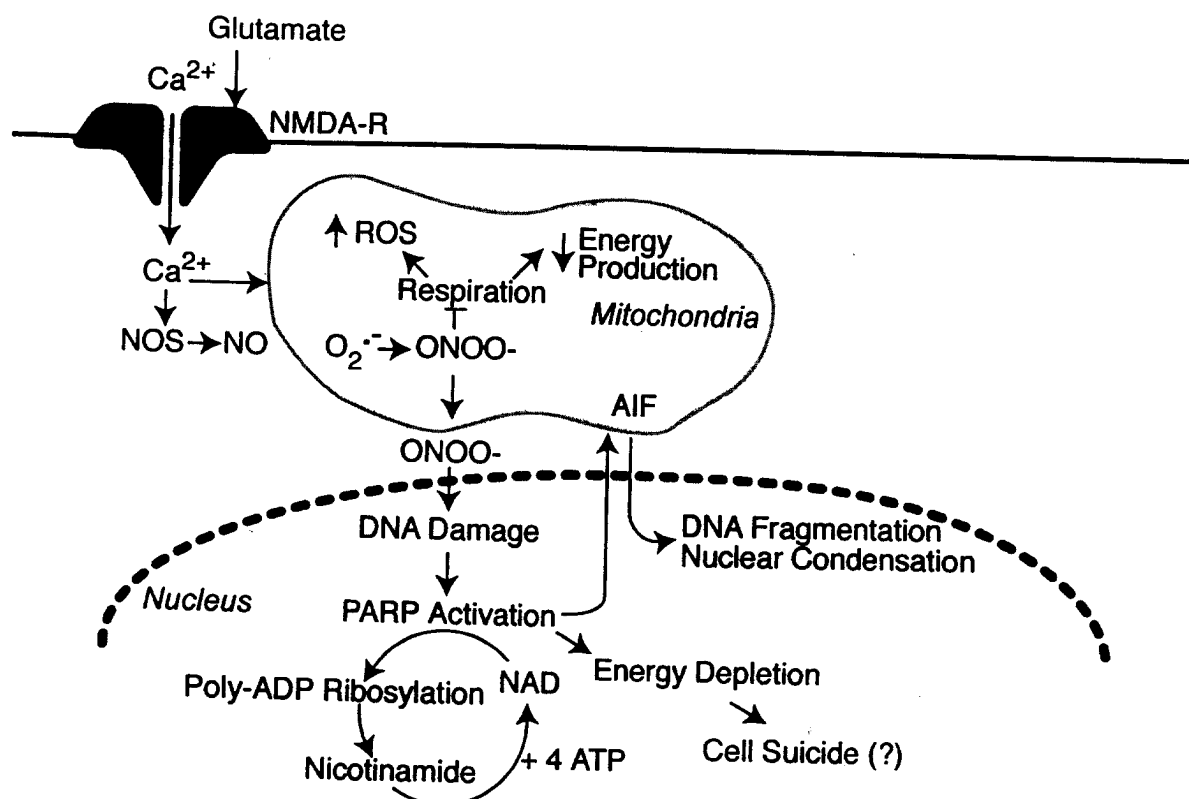


Fig. 1. Model of PARP-1-mediated excitotoxicity. NMDA receptor activation results in ROS and peroxynitrite (ONOO^-) formation. ONOO^- inhibits respiration leading to increased ROS and damages DNA-activating PARP-1. NAD is consumed to poly(ADP-ribosylate) proteins. PARP-1 activation results in AIF release from the mitochondria. Nuclear shrinkage and DNA fragmentation are observed when AIF enters the nucleus.

activation of PARP-1 can lead to massive NAD^+ utilization, and changes in the cellular NAD^+ and ATP levels led Berger and Okamoto to propose that consumption of NAD^+ due to DNA damage and activation of PARP-1 can affect cellular energetics and function ultimately leading to cell death due to excessive energy consumption (Berger and Berger, 1986; Berger *et al.*, 1983). Studies in various cell and animal models have consistently observed depletion of NAD^+ and ATP that is blocked by PARP-1 inhibition or deletion. However, none of these studies address whether loss of cellular energetics is the cause of excitotoxic cell death or is simply a biomarker for PARP-1 activation. This is a difficult question to experimentally address. Addition of precursors such as creatine, can provide protection but these agents activate multiple mitochondrial and cellular pathways that may protect cells in a manner that is independent from the signal cascade triggered by PARP-1-mediated NAD^+ loss. In an experimental stroke model recent data indicate that preservation of energy stores in PARP-1 knockout mice is not the mechanism underlying the reduction in infarct volume (Goto *et al.*, 2002). The time-course and severity of the apparent diffusion coefficient (ADC), an *in vivo* measure of cellular energy stores, is not altered in PARP-1 knockout brains compared with wild-type brains, despite the fact that the PARP-1 knockout animals had smaller infarct volumes compared with wild type animals (Goto *et al.*, 2002). Thus, energy depletion alone might not be sufficient to mediate PARP-1-dependent cell death. Recently we identified a role for AIF as a downstream-signaling molecule in the PARP-1-dependent cell death but how PARP-1 triggers AIF release and cell death is not yet known (Fig. 1).

APOPTOSIS-INDUCING FACTOR (AIF)

Recent and emerging data indicate that AIF plays an important role in excitotoxic neuronal death. Mammalian AIF is a 67-kDa protein containing an *N*-terminal mitochondrial localization sequence and a large C-terminal with homology to bacterial oxidoreductases (Susin *et al.*, 1999). AIF is evolutionarily conserved with homologs found in invertebrates, nematodes, fungi, and plants. AIF can stably bind FAD, which places AIF in the category of flavoproteins. AIF also displays NAD(P)H oxidase as well as monodehydroascorbate reductase activities (Miramar *et al.*, 2001). The overall crystal structure of mature mouse AIF has been recently resolved at 2.0 Å resolution. AIF displays a glutathione-reductase-like fold, with an FAD-binding domain, an NADH-binding domain, and a C-terminal domain that bears a small AIF-specific insertion (509-559) not found in glutathione reductase. The

amino acids interacting with FAD and NADH have been mapped precisely, and the mutants E313A and K176A have been shown to reduce FAD binding (Mate *et al.*, 2002). Mutational analysis reveals that regardless of the presence or the absence of NAD(P)H and/or FAD (which is the essential prosthetic group of the oxidoreductase), AIF can induce nuclear apoptosis (Loeffler *et al.*, 2001; Miramar *et al.*, 2001). These data indicate that the oxidoreductase function of AIF is not required for its apoptogenic action.

The normal physiologic activity of AIF is not known. Recent data from the Harlequin mice in which expression of AIF 67 kDa is reduced 80% due to a proviral insertion into AIF genomic DNA suggests that AIF might participate in scavenging ROS (Klein *et al.*, 2002). The putative redox reaction catalyzed by AIF in mitochondria is also not known. On the basis of its similarity to prokaryotic oxidoreductases, it is possible that AIF might interact with the cytochrome *bc*1 complex, which catalyzes the electron transfer from ubiquinone to cytochrome *c* in the mitochondrial respiratory chain (Mate *et al.*, 2002). Theoretically, AIF could catalyze the reduction of cytochrome *c* in the presence of NADH *in vitro* (Miramar *et al.*, 2001).

Following exposure of a cell to cytotoxic insults, AIF translocates to the cytosol and then the nucleus, where it induces peripheral chromatin condensation and high-molecular-weight (50 kb) DNA fragmentation. Translocation of AIF to the nucleus appears to be a general feature of apoptosis in mammalian cells (Cande *et al.*, 2002) but whether it is a primary execution step or a secondary participatory step is dependent on the death signal and cell type. Confirmation of the death effector role for AIF when released from its normal mitochondrial compartment, are the observations resulting from forced cytosolic expression of AIF in the absence of an external death signal (Loeffler *et al.*, 2001). The crystal structure of human AIF revealed the presence of a strong positive electrostatic potential at the AIF surface. AIF colocalizes with DNA at an early stage of nuclear morphological changes, as indicated by electron microscopy. The electrostatic interaction between AIF and DNA is independent of the DNA sequence. Structure-based mutagenesis showed that DNA-binding defective mutants of AIF fail to induce cell death (Ye *et al.*, 2002). This suggests that DNA-binding by AIF is required for its apoptogenic function in the nuclear compartment. Two of the mutants that completely blocked the capacity of AIF to interact with DNA and to induce chromatin condensation (K255A, R265A, and K510A/K518A), still retained NADH oxidase activity (Ye *et al.*, 2002), thus confirming that the oxidoreductase and apoptosis-inducing activities of AIF can be fully dissociated.

How AIF induces chromatin condensation and DNA fragmentation remains a mystery. There are several possible scenarios. AIF could itself have some cryptic nuclease activity that has not yet been observed. The interaction of AIF with DNA may increase the susceptibility of DNA to latent endogenous nucleases. AIF might recruit nucleases to induce partial chromatin fragmentation.

The mitochondrial-nuclear translocation of AIF is caspase-independent in some types of cell death (Cregan *et al.*, 2002; Susin *et al.*, 1999; Yu *et al.*, 2002) as treatment with caspase inhibitors fails to block AIF translocation and cell death (Yu *et al.*, 2002). Additionally, translocation of AIF can be observed in vitro in cells in which there is no caspase activation, owing to genetic deletion of Apaf-1, caspase-9 or caspase-3 (Cregan *et al.*, 2002; Susin *et al.*, 2000). Similar observations have been obtained in *Apaf-1*^{-/-}, *caspase-9*^{-/-} or *caspase-3*^{-/-} embryoid bodies, in which AIF translocates during cavitation (Joza *et al.*, 2001). Microinjection or transfection of *Apaf-1*^{-/-}, *caspase-9*^{-/-}, or *caspase-3*^{-/-} cells with recombinant AIF protein or transient expression of AIF, also induces cell death without caspase activation. Features of classic apoptosis, such as phosphatidylserine exposure, partial chromatin condensation, and cellular shrinkage are still observed in these cells (Loeffler *et al.*, 2001; Susin *et al.*, 2000). In vitro, both purified natural AIF and recombinant AIF alter the structure of chromatin resulting in large-scale DNA fragmentation in purified nuclei. This "nuclear apoptosis" cannot be prevented by caspase inhibitors (Susin *et al.*, 2000; Yu *et al.*, 2002). While these data strongly support the notion that AIF acts as a caspase independent death effector, there also exists data for an interaction between AIF and the caspase cascade. Activated caspases (caspase-8 and caspase-2) and the caspase-activated protein *t*-Bid can trigger the release of AIF from mitochondria (Lassus *et al.*, 2002; Robertson *et al.*, 2002; Zamzami *et al.*, 2000). In HeLa and Jurkat cell lines treated with staurosporine or actinomycin D, mitochondrial release of AIF is suppressed or delayed by caspase inhibitors (Arnoult *et al.*, 2001). Genetic data obtained in *C. elegans* also suggest that AIF operates partially in a caspase-dependent fashion. Heat-shock inducible expression of Egl-1 causes the mitochondrial release of green fluorescent protein (GFP)-tagged AIF in all *C. elegans* wild-type embryos, but *Ced-3* loss-of-function mutants there is a >80% inhibition of AIF release (Wang *et al.*, 2002). Transgenic expression of AIF and endonuclease G kills >60% of wild-type cells in *C. elegans*, but in *Ced3*-deficient animals cell death is reduced to 28%. These data suggest that, although AIF and endonuclease G can function in a caspase-independent fashion, AIF and endonuclease G are more efficient in a *Ced-3*-positive background (Wang

et al., 2002). Taken together the published literature supports both caspase-independent or caspase-dependent mechanisms of AIF release from the mitochondria that are dependent on cell type and the lethal stimulus.

In the CNS AIF may be particularly important in mediating neurotoxicity due to both acute and chronic (neurodegenerative) diseases. Acute neurotoxicity can be induced by trauma, hypoglycemia, or transient ischemia. The translocation of AIF has been observed in several experimental models of neurotoxicity such as the death of photoreceptors induced by retinal detachment (Hisatomi *et al.*, 2002), neuronal cell death induced in vivo by brain trauma (Zhang *et al.*, 2002) and death of cortical neurons induced in vitro by exposure to heat-inactivated *Streptococcus pneumoniae* (Braun *et al.*, 2001), hydrogen peroxide, peroxynitrite (Zhang *et al.*, 2002), the topoisomerase I inhibitor camptothecin, infection with a p53-expressing adenovirus (Cregan *et al.*, 2002), or the excitotoxin NMDA (Yu *et al.*, 2002). In a model of neurotrauma, the translocation of AIF in selected brain areas could be correlated with genomic DNA degradation to ~50 kb fragments (which is a hallmark of AIF-mediated nuclear apoptosis) (Zhang *et al.*, 2002). We have shown that DNA-damage-induced AIF translocation and apoptosis depends on the presence of p53 and its transcriptional target Bax (Cregan *et al.*, 2002). Under excitotoxic conditions we have shown that NMDA-induced release of AIF, is PARP-dependent. Microinjection of a neutralizing antibody recognizing a surface-exposed domain of AIF prevents cell death (Cregan *et al.*, 2002; Yu *et al.*, 2002) but caspase inhibition alone has no beneficial effect on cell survival (Braun *et al.*, 2001; Yu *et al.*, 2002; Zhang *et al.*, 2002). Assuming that the anti-AIF antibody has no additional effects this suggests that AIF contributes to neurotoxicity. Taken together these data suggest that neurons are susceptible to both caspase-dependent and -independent death programs and that AIF can participate in both. In NMDA excitotoxicity the death program runs in a serial manner (Fig. 2). Blocking NOS, PARP, or AIF prevents the downstream events from occurring and prevents death. Caspase activation is a consequence of AIF release and does not contribute to cell death as inhibition of caspases does not produce cell survival. In neurotoxicity due to p53 activation following DNA-damage parallel pathways are activated. The caspase pathway is primary but blocking this pathway merely delays cell death. A parallel p53 pathway also activates the AIF pathway. In order to elicit neuroprotection both parallel death programs, the caspase and AIF pathways, must be blocked. This type of parallel death machinery might be important in non-NMDA excitotoxicity elicited by AMPA or kainate.

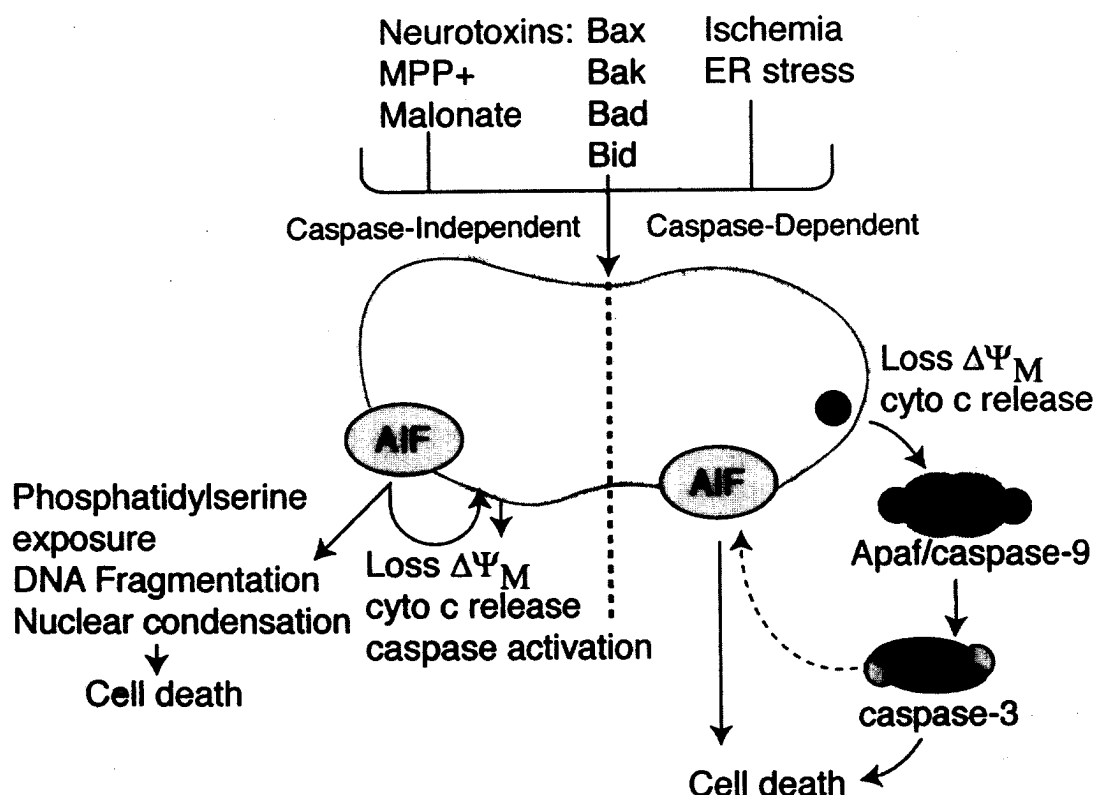


Fig. 2. AIF in caspase-independent and caspase-dependent cell death. Injury to the nervous system can elicit both caspase-dependent and -independent cell death that is both stimulus and cell type dependent. AIF can direct kill cells without caspase participation. In other death pathways caspases are active and are sufficient to kill the cell but can also recruit AIF to the death machinery. Additionally, some death stimuli, such as p53, activate AIF and caspase pathways simultaneously.

SUMMARY

In the CNS, injury is mediated by a carefully choreographed series of events initially triggered by calcium influx through the NMDA glutamate receptor activating nNOS and mitochondrial respiration (Fig. 3). The production of NO and superoxide anion result in the formation of peroxynitrite, which can diffuse from the mitochondria to damage various cellular constituents. In the nucleus the nicks in DNA induced by peroxynitrite activate the enzyme PARP-1 which consumes NAD in the process of generating PAR and modifying proteins with PAR. Activation of PARP-1 signals to the mitochondria and AIF is released. Translocation of AIF to the nucleus results in large-scale DNA fragmentation and nuclear shrinkage. This is likely the final commitment point to cell death. Genetic deletion studies and pharmacologic inhibition studies indicate that this pathway is critically important in several models of neurologic injury including models of stroke and Parkinson's disease. However, serial pathways may also be activated in some neurologic diseases.

It is important to identify all pathways and order the sequence of events to better understand the signal cascades that result in neuronal death. It is the hope that a better understanding of these events will lead to identification of new target molecules to treat patients with neurologic disease.

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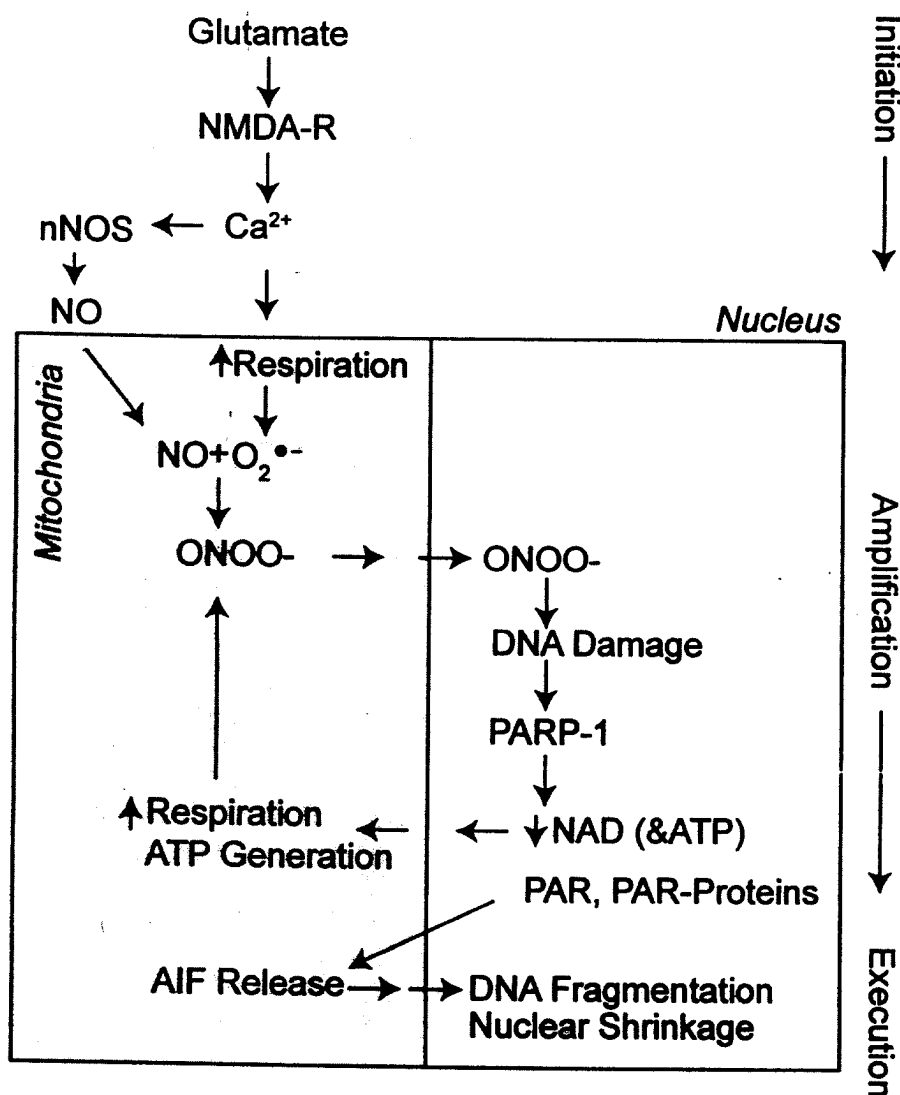


Fig. 3. Schematic of the ROS, PARP-1, AIF death program in excitotoxicity. In the initiation phase of neurotoxicity, activation of NMDA glutamate receptors leads to increased intracellular calcium that activates neuronal nitric oxide synthase (nNOS) producing nitric oxide (NO). Neuronal activation also results in increased oxidative phosphorylation and subsequently increased superoxide anion production in the mitochondria. Superoxide is not membrane permeable and resides largely in the mitochondria where it is generated. NO and superoxide anion react to form the potent oxidant, peroxynitrite. Peroxynitrite generation can trigger an amplification phase of neurotoxicity by attacking mitochondrial proteins in the electron transport chain including complex I and IV as well as the superoxide scavenging enzyme, manganese superoxide dismutase (MnSOD). This initiates a viscous cycle of peroxynitrite generation through sustained superoxide anion generation. Peroxynitrite is membrane permeable and can move to the nucleus triggering DNA strand breaks. Damaged DNA activates poly(ADP-ribose) polymerase (PARP) resulting in the synthesis of PAR polymers, ribosylation of proteins, and consumption of NAD and ATP. These PARP dependent events signal the mitochondria to release apoptosis inducing factor (AIF) that translocates to the nucleus. In the nucleus AIF triggers large scale DNA fragmentation and nuclear condensation. These nuclear changes in neurons are likely the final commitment and execution point in the neurotoxic cascade. Subsequent to these events cytochrome c is released and caspases are activated. Blocking these events does not prevent NO/PARP-dependent neurotoxicity but may be important in preparation of the corpse and the degradation of the cell.

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Neuronal Apoptosis: BH3-Only Proteins the Real Killers?

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At present there is a poor understanding of the events that lead up to neuronal apoptosis that occurs in neurodegenerative diseases and following acute ischemic episodes. Apoptosis is critical for the elimination of unwanted neurons within the developing nervous system. The Bcl-2 family of proteins contains pro- and anti-apoptotic proteins that regulate the mitochondrial pathway of apoptosis. There is increasing interest in a subfamily of the Bcl-2 family, the BH3-only proteins, and their pro-apoptotic effects within neurons. Recently ischemic and seizure-induced neuronal injury has been shown to result in the activation of the BH3-only protein, Bid. This protein is cleaved and the truncated protein (tBid) translocates to the mitochondria. The translocation of tBid to the mitochondria is associated with the activation of outer mitochondrial membrane proteins Bax/Bak and the release of cytochrome C from the mitochondria. ER stress also has been implicated as a factor for the induction of apoptosis in ischemic neuronal injury. The induction of ER stress in hippocampal neurons has been shown to activate expression of *bb3/PUMA*, a member of the BH3-only gene family. Activation of PUMA is associated with the activation and clustering of the pro-apoptotic Bcl-2 family member Bax and the loss of cytochrome C from the mitochondria.

KEY WORDS: BH3-only proteins; ischemia; ER stress; Bcl-2 family proteins; mitochondria; apoptosis; necrosis.

INTRODUCTION

Excitotoxic neuron death has been implicated in the pathogenesis of ischemic, traumatic, and epileptic brain injury (Choi, 1994). Following prolonged glutamate receptor overactivation there is extensive necrotic cell death. This is characterized by increased free radical production, a collapse in the mitochondrial membrane potential ($\Delta\psi_m$), disruption of Ca^{2+} homeostasis, ATP depletion, and an increase in cellular volume (Ankarcrona *et al.*, 1995; Choi, 1987; Tymianski *et al.*, 1993). However, when glutamate receptor activation is only transient, a more delayed cell death may result (Ankarcrona *et al.*, 1995; Budd *et al.*, 2000; Luetjens *et al.*, 2000; Ward *et al.*, 2000). This delayed cell death is associated with a release of cytochrome C from the mitochondria and a collapse of the $\Delta\psi_m$ (Budd *et al.*, 2000; Lankiewicz *et al.*, 2000; Luetjens

et al., 2000; Ward *et al.*, 2000). The molecular mechanism of the mitochondrial cytochrome C release during excitotoxic neuronal cell death remains unresolved.

In the "classical" mitochondrial apoptosis signalling pathway, the release of cytochrome C requires the pro-apoptotic Bcl-2 family members Bax or Bak (Wei *et al.*, 2001). These proteins are believed to form pores that make the outer mitochondrial membrane sufficiently permeable for the release of intermembrane proteins, which include cytochrome C (Kuwana *et al.*, 2002). For this to occur, Bax and Bak must undergo conformational changes and enter into the outer mitochondrial membrane (Esques *et al.*, 1998; Goping *et al.*, 1998). The transcriptional induction or the posttranslational activation of Bcl-2-homology domain-3 (BH3)-only proteins is required to trigger the activation of Bax and Bak (Huang and Strasser, 2000), which can be antagonized by the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL.

BH3-ONLY PROTEINS

The BH3-only proteins are a subfamily of the Bcl-2 protein family that are essential initiators of programmed

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Table I. Mammalian BH3-Only Proteins: Stimuli and Regulation

BH3-only proteins	Stimulus; upstream regulators	Mode of activation	Reference
Bad	Growth factor withdrawal; seizure-induced cell death	Phosphorylation	Zha <i>et al.</i> , 1996; Henshall <i>et al.</i> , 2001
Bid	Ischemia; seizure-induced cell death	Lipid modification, Proteolytic cleavage	Desagher <i>et al.</i> , 1999; Wei <i>et al.</i> , 2001; Plesnila <i>et al.</i> , 2001
Bik	Growth factor withdrawal; FKHRL 1, JNK	Phosphorylation	Biswas and Greene, 2002
Bim		Transcriptional induction, Phosphorylation, Translocation	Puthalakath <i>et al.</i> , 1999; Putcha <i>et al.</i> , 2001
Blk	Anoikis(= matrix detachment)	Phosphorylation	Hedge <i>et al.</i> , 1998
Bmf		Phosphorylation, Translocation	Puthalakath <i>et al.</i> , 2001
BNIP3	Hypoxia	Transcriptional induction	Yasuda <i>et al.</i> , 1998
NIX	Hypoxia	Transcriptional induction	Yasuda <i>et al.</i> , 1998
Hrk	JNK	Transcriptional induction	Inohara <i>et al.</i> , 1998
PUMA	p53; ER stress	Transcriptional induction	Yu <i>et al.</i> , 2001
Noxa	p53; Hypoxia	Transcriptional induction	Oda <i>et al.</i> , 2000; Kim <i>et al.</i> , 2004
Spike		Transcriptional induction	Mund <i>et al.</i> , 2003

cell death through the activation of Bax and Bak. All the members of this protein family contain a short amino acid (9-16 amino acids) BH3- domain, however they do not possess a very strong structural homology (Huang and Strasser, 2000). The BH3 domain is essential for the binding of these BH3-only proteins to both pro- and antiapoptotic members of the Bcl-2 family. In *C. elegans* a single BH3-only protein, EGL-1, is required for the initiation of programmed cell death (Conradt and Horvitz, 1998), however, in mammals there are at least 12 BH3-only proteins (see Table I). The diversity of these BH3-only proteins is reflected in the different modes of activation. They operate by either blocking the actions of the anti-apoptotic proteins (Bcl-2 and Bcl-xL), thereby facilitating apoptosis, or by promoting apoptosis through the activation of pro-apoptotic proteins (Bax and Bak).

Excitotoxic Injury

In recent years it is becoming more evident that BH3-only proteins are integrally involved with the apoptotic neuronal death cascade. Neurons from mice deficient of the BH3-only protein Bid have been shown to be resistant to ischemic injury in vivo, as well as hypoxic and excitotoxic injury in vitro (Plesnila *et al.*, 2001). The activation of Bid and the subsequent cleavage of Bid to the truncated form (tBid) is an essential component of most forms of receptor-mediated apoptosis (Li *et al.*, 1998; Luo *et al.*, 1998). During an ischemic-episode- or seizure-induced

neuronal death, Bid is truncated to its 15-kDa form that targets the mitochondria (Henshall *et al.*, 2001; Plesnila *et al.*, 2001). This cleavage of Bid has been shown to occur through caspase-8 (Li *et al.*, 1998; Luo *et al.*, 1998). tBid is then translocated to the mitochondria, where it activates Bax or Bak and induces the release of cytochrome C from the mitochondria (Eskes *et al.*, 2000; Wei *et al.*, 2000). As caspase activation may only be very marginal in excitotoxic neuronal apoptosis (Armstrong *et al.*, 1997; Budd *et al.*, 2000; Lankiewicz *et al.*, 2000) this suggests that the activation of Bid may trigger excitotoxic neuronal injury through another pathway (Chen *et al.*, 2001; Stoka *et al.*, 2001). There is also increasing evidence that Bim may play a significant role in the regulation of neuronal vulnerability in seizure-induced neuronal injury (Shinoda *et al.*, in press)

ER Stress

It has been suggested that ER stress may also contribute to the induction of neuronal apoptosis injury following ischemia (Paschen and Frandsen, 2001). ER stress is the term given to any condition that results in the accumulation of unfolded or misfolded proteins within the ER lumen (Kauffman, 1999). In rodent cells, ER-stress-induced cell death has been shown to involve the activation of ER-resident caspase-12, which subsequently activates executioner caspases such as caspase-3 (Nakagawa and Yuan, 2000; Nakagawa *et al.*, 2000). However, there is

increasing evidence that ER stress may activate the mitochondrial apoptotic pathway that results in the release of cytochrome C from the mitochondria (Annis *et al.*, 2001; Häcki *et al.*, 2000; Wei *et al.*, 2001). This requires an increase in the permeability of the outer mitochondrial membrane that is triggered by Bax and Bak (Desagher and Martinou, 2000; Wei *et al.*, 2001). In a recent study, Reimertz *et al.* (2003) used tunicamycin to induce ER stress in hippocampal neurons. Tunicamycin prevents protein glycosylation and results in the buildup of malformed proteins within the lumen of the ER. This in turn results in the characteristic unfolded protein response (UPR). Following tunicamycin there is an increase in the expression of a number of different genes that are typically involved in the UPR response, such as *BIP* and *GRP 94* (for a review see Kaufman *et al.*, 1999). These molecular chaperones help relieve ER stress by promoting protein folding and keeping proteins in a folding competent state. Following ER stress induced by tunicamycin, there is induction of the *bbc3/PUMA* protein and subsequent activation of the mitochondrial apoptosis pathway indicated by the release of cytochrome C from the mitochondria (Reimertz *et al.*, 2003). In addition to ER stress, *Bbc3/PUMA* is also activated after transient forebrain ischemia. Furthermore, overexpression of *Bbc3/PUMA* is sufficient to trigger apoptosis in neuronal cells, and cells deficient in *bbc3/PUMA* showed dramatically reduced apoptosis in response to ER stress (Reimertz *et al.*, 2003).

It is apparent that the BH3-only proteins play an integral part in the apoptotic pathways that are associated with ischemic and seizure-induced neuronal injury. Through a more detailed understanding of these pathways we will gain new insight into the regulatory pathways that control neuronal injury and through this we may find the new targets for future drug development.

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Protective Roles of CNS Mitochondria

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Mitochondria benefit their host cells by generating ATP, detoxifying oxygen, maintaining cellular redox potential, and detoxifying reactive oxygen species and xenobiotics. These beneficial roles are in stark contrast to mitochondrial participation in both necrotic and apoptotic degenerative pathways. However, cellular stresses do not always result in deleterious mitochondrial changes. Decreases in the calcium sensitivity of the permeability transition may be initial mitochondrial responses to stress that act to preserve mitochondrial function and prolong normal functioning of the host cell.

KEY WORDS: Permeability transition; calcium sensitivity; Huntington's disease; regional vulnerability; neurodegeneration.

With much focus upon the role of mitochondria in apoptotic signaling and the permeability transition in acute necrotic injury, the roles mitochondria play in generating energy, maintaining homeostasis and protecting cells from injury may appear as secondary or lesser functions. However, the role of mitochondria in preserving cell health is clearly the more dominant function. First and foremost is the principal role of mitochondria in generating ATP. Concomitant with generating ATP, reducing molecular oxygen through the electron transport chain prevents its spurious involvement in oxidation of other important cellular constituents, i.e., proteins, lipids, or oligonucleotides (Skulachev, 1996). Electron transport and oxidative phosphorylation harness the high redox potential of O₂, transforming it into the high-energy phosphate bond of ATP. With O₂ as substrate, its flux becomes part of a highly regulated set of reactions, and thus it is less likely to participate in spurious, harmful oxidative activity. Residual reactive oxygen species are largely controlled either by transmutase reactions or by reducing their production by increasing O₂ utilization through the electron transport

chain. Mild uncoupling, or even induction of the permeability transition, could serve to temporarily depolarize mitochondria and decrease production of reactive oxygen species. Thus, mitochondria benefit the cell by both producing energy efficiently and by largely detoxifying oxygen (Skulachev, 1996).

Mitochondria also function to maintain cellular homeostasis by sequestering calcium (Nicholls, 1985). Mitochondrial membrane potential is a potent driving force for moving Ca²⁺ up a concentration gradient into the mitochondrial matrix. Once inside, free Ca²⁺ is controlled by its solubility in association with phosphate, permitting temporary storage of Ca²⁺ in a relatively inactive form (Chalmers and Nicholls, 2003). Mitochondria act as a transient Ca²⁺ sink during rapid events that increase cytosolic Ca²⁺, such as the neuronal action potential (Werth and Thayer, 1994), and during longer lasting events associated with Ca²⁺ waves (Landgraf *et al.*, 2004). Under stressful conditions, when neurons are challenged by overexposure to the excitotoxic neurotransmitter glutamate, mitochondrial sequestering of Ca²⁺ may figure critically in the ability of the neuron to prevent necrosis. Several recent studies of the often destructive mitochondrial permeability transition have revealed new ways in which mitochondria may actually be acting to preserve mitochondrial and cellular function.

In isolated mitochondrial preparations, bolus addition of a substantial Ca²⁺ load or repeated additions of smaller Ca²⁺ doses eventually leads to mitochondrial

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depolarization, swelling, and an eventual inability of mitochondria to sequester the calcium. Evidence for the occurrence of this permeability transition *in situ* has been gathered from astrocytes, neurons, and a plethora of non-neuronal cells (Dubinsky and Levi, 1998; Jordan *et al.*, 2003; Kristal and Dubinsky, 1997; White and Reynolds, 1996). When neuronal cytosolic Ca^{2+} is elevated with an excitotoxic dose of glutamate, mitochondria depolarize in a manner that may or may not be sensitive to inhibitors of the permeability transition (Brustovetsky and Dubinsky, 2000b; Budd *et al.*, 2000; Reynolds, 1999).

Ambiguity surrounding a role of the permeability transition in excitotoxicity may lie in the variety of responses receiving that name and in the differential susceptibility of mitochondria from different neuronal populations. The conductance pathways opened by mPT may vary. In single-channel recordings, large conductance mitochondrial channels may be activated spontaneously or by peptides, Bcl-xL, or Ca^{2+} (Jonas *et al.*, 2003; Kushnareva *et al.*, 2001; Muro *et al.*, 2003). Any or all of these could contribute to events ascribed to permeability transition. In isolated mitochondrial preparations, Ca^{2+} may trigger a high conductance response, associated with simultaneous depolarization and swelling (Brustovetsky and Dubinsky, 2000a,b). While mitochondria may recover from such an event, it often results in mitochondrial disintegration and may be part of a regulated autophagic pathway (Lemasters *et al.*, 2002). Ca^{2+} may also activate a limited permeability that only depolarizes mitochondria, without causing swelling (Brustovetsky and Dubinsky, 2000a,b). Initiated under conditions of limited substrate availability, isolated CNS mitochondria respond to external Ca^{2+} with such a sustained depolarization. This depolarization drastically reduces the driving force for Ca^{2+} influx, limiting the mitochondria's ability to sequester Ca^{2+} (Brustovetsky and Dubinsky, 2000a,b). Such a response should protect mitochondria against more severe damage. Indirectly it protects the neuron from the eventuality of a compromised metabolism, preserving mitochondrial integrity for future ATP generation. Seen from the mitochondria's point of view, this limited permeability pathway, which may be a component of or precursor to the high conductance mPT, is an attempt to prevent the latter from occurring. Preventing Ca^{2+} entry precludes accumulation of matrix free Ca^{2+} to levels sufficient for induction of a high conductance mPT. Among the heterogeneous responses of cultured hippocampal neurons, mitochondrial depolarization accompanying glutamate-induced sustained increases in intracellular calcium may reflect opening of such low conductance pathways (Fig. 1). In such cases, FCCP addition to fully depolarize mitochondria and unload accumu-

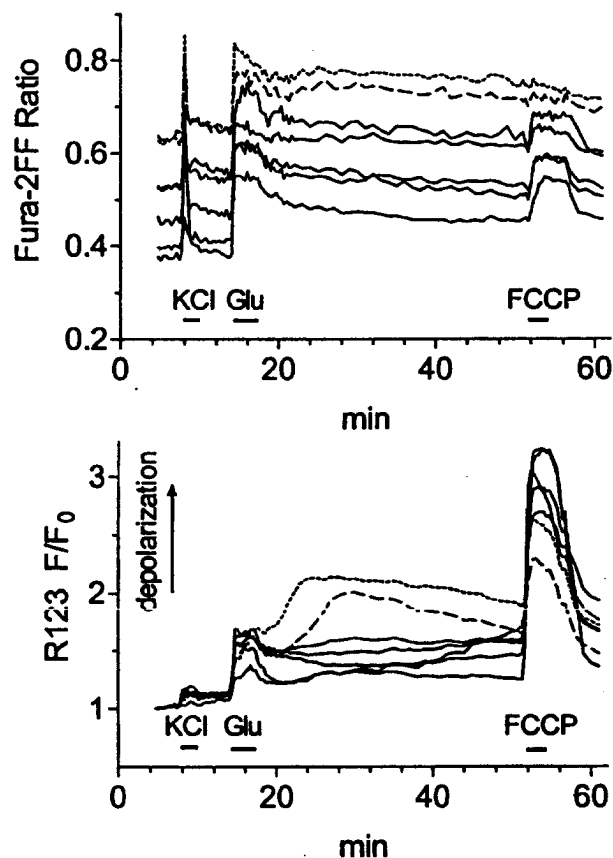


Fig. 1. Cultured hippocampal neurons loaded with the low affinity Ca^{2+} dye, fura-2FF, and the mitochondrial membrane potential sensitive dye, R123, display mixed reactions to 500 μM glutamate. Neurons with the most depolarized mitochondria display the highest sustained calcium levels. Mitochondrial depolarization with FCCP reveals an absence of mitochondrially stored calcium. (Reprinted with permission from *J. Neurosci.* 20, 103)

lated calcium results in no further increase in cytosolic Ca^{2+} (Brustovetsky and Dubinsky, 2000a,b). In a similar manner, chemically induced transient mitochondrial depolarization of cultured cortical neurons is neuroprotective (Stout *et al.*, 1998). In cerebellar cultures, similar mitochondrial depolarization can prevent glutamate-induced delayed calcium deregulation, a precursor to cell death (Nicholls and Budd, 1998). Admittedly, while depolarization via activation of a low conductance permeability may be protective of the mitochondria, it may not always result in endogenous neuroprotection. Elevated cytosolic Ca^{2+} can initiate a variety of other deleterious pathways, which results in cell death. However, preserving mitochondrial integrity may result in these other pathways, leading to a slower and more tidy apoptotic process, fueled by mitochondrial generated ATP, rather than an acute necrotic demise.

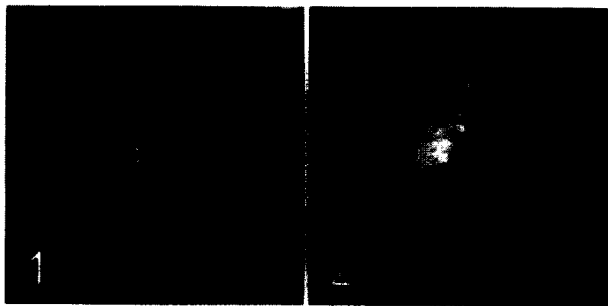


Fig. 2. Changes in mitochondrial morphology often associated with excitotoxicity. Cultured hippocampal neuron stained with mitotracker green (1) initially and (2) 20 min after 5 min of 500 μ M glutamate.

Variability in regional brain mitochondria may also contribute to the controversy regarding whether or not the mPT participates in excitotoxicity. Strong arguments have been made against its participation in glutamate-induced delayed calcium deregulation in cultured cerebellar granule cells (Nicholls and Budd, 1998). However, isolated cerebellar mitochondria are less sensitive to Ca^{2+} -induced swelling than mitochondria from the cortex or hippocampus (Friberg *et al.*, 1999). Hippocampal neurons variably display morphological changes in mitochondria

after excitotoxic glutamate exposure (Fig. 2) (Dubinsky and Levi, 1998), consistent with the high sensitivity of hippocampal mitochondria to Ca^{2+} -induced swelling (Friberg *et al.*, 1999). Similarly, isolated rat striatal mitochondria appear more susceptible to Ca^{2+} -induced depolarization and swelling than cortical mitochondria (Brustovetsky *et al.*, 2003). Such differential sensitivity may contribute to the initially greater susceptibility of striatal neurons in degenerative diseases such as Huntington's disease. To address this question, we examined regional brain mitochondria from a very slowly progressing mouse model of HD in which long polyglutamine expansions have been placed in exon 1 of the mouse Huntington gene (Wheeler *et al.*, 2000). Indeed, when striatal and cortical mitochondria from Q111 mutant huntingtin knock-in mice of various ages were examined, the initially more susceptible striatal mitochondria changed with increasing age in a polyglutamine-dependent manner (Fig. 3). Striatal mitochondrial Ca^{2+} sensitivity decreased until these mitochondria became equally sensitive to the cortical mitochondria. The shift in Ca^{2+} sensitivity occurred very early in the disease progression, at the time that nuclear localization of mutant huntingtin first occurred (Wheeler *et al.*, 2000). Thus, in the early stages of disease progression, the

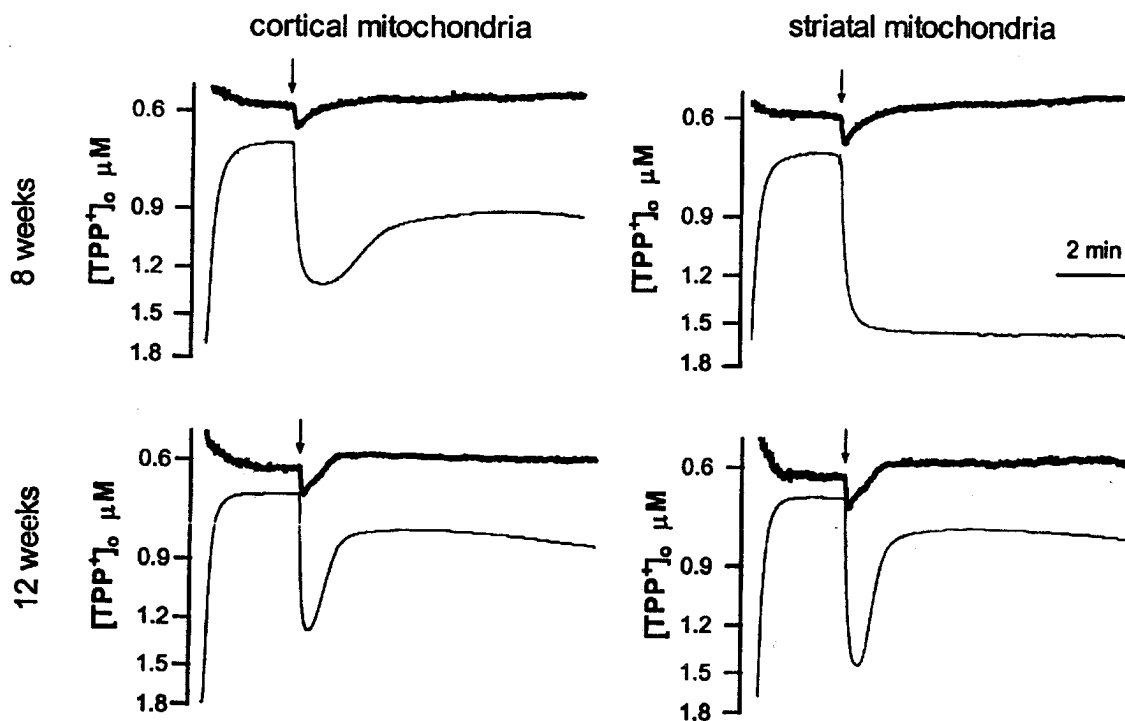


Fig. 3. Isolated cortical and striatal mitochondria from Q111 mice 8 and 12 weeks of age. Mitochondrial membrane potential was measured by the uptake of TPP^+ (thin lines) and swelling was monitored by light scattering (thick lines) (Brustovetsky *et al.*, 2003); 0.3 μ mol Ca^{2+} per mg mitochondrial protein was added at the arrows. In older Q111 mice, striatal mitochondria retain the same Ca^{2+} sensitivity as observed at 12 weeks.

striatal mitochondria became more resistant to induction of permeability transition. This compensatory response again demonstrates a mitochondrial propensity for self-preservation. A higher Ca^{2+} threshold will make neurons more tolerant of increases in intracellular Ca^{2+} . However, this adaptive strategy may only work in the short term. If uncoupling and permeability transition are indeed safety valves or ways for cells to detoxify extra oxygen by maximally running electron transport (Skulachev, 1996), then by raising the threshold for onset of permeability transition, neurons may increase their risk for cumulative oxidative damage. As with any stressed tissue that mounts a compensatory response, overall susceptibility of the entire cell to other degenerative processes may be increased.

Similar adaptive mitochondrial behavior has been previously reported in precancerous liver cells of rats fed the carcinogen 2-acetylaminofluorene (Klohn *et al.*, 2003). Liver mitochondria harvested from animals on this diet for 3–4 weeks had a greatly decreased sensitivity to Ca^{2+} activation of mPT. While the epigenetic process leading to this increased resistance remains unknown, the mitochondrial response is clearly one of self-preservation, both for the mitochondria and for the immediate livelihood of the hepatocytes. Their eventual progression into neoplastic cells, resistant to cell death, may be a logical extension of the initially protective response.

Thus, in addition to their primary roles in energy generation, oxygen fixation, and calcium sequestration, mitochondria may adapt to locally stressful conditions with subtle protective behaviors. Depolarization resulting from uncoupling or Ca^{2+} -activated pathways may prevent mitochondrial disintegration and relieve the cell of excess oxygen (Skulachev, 1996). Shifting a cell's sensitivity to Ca^{2+} induction of a high conductance permeability transition may avoid acute necrosis, allowing cellular homeostasis to adapt to stressful conditions. Mitochondrial self-protection may be an early initial response to the stresses of elevated cytosolic calcium. Such adaptive behaviors

may benefit the mitochondria themselves, their host cells, and the whole tissue.

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Ammonia Neurotoxicity and the Mitochondrial Permeability Transition

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Ammonia is a neurotoxin that predominantly affects astrocytes. Disturbed mitochondrial function and oxidative stress, factors implicated in the induction of the mitochondrial permeability transition (MPT), appear to be involved in the mechanism of ammonia neurotoxicity. We have recently shown that ammonia induces the MPT in cultured astrocytes. To elucidate the mechanisms of the MPT, we examined the role of oxidative stress and glutamine, a byproduct of ammonia metabolism. The ammonia-induced MPT was blocked by antioxidants, suggesting a causal role of oxidative stress. Direct application of glutamine (4.5–7.0 mM) to cultured astrocytes increased free radical production and induced the MPT. Treatment of astrocytes with the mitochondrial glutaminase inhibitor, 6-diazo-5-oxo-L-norleucine, completely blocked free radical formation and the MPT, suggesting that high ammonia concentrations in mitochondria resulting from glutamine hydrolysis may be responsible for the effects of glutamine. These studies suggest that oxidative stress and glutamine play major roles in the induction of the MPT associated with ammonia neurotoxicity.

KEY WORDS: Ammonia; astrocytes; glutamine; mitochondrial permeability transition; oxidative stress.

INTRODUCTION

Ammonia is a neurotoxin that has been strongly implicated in the pathogenesis of hepatic encephalopathy (HE), an important cause of morbidity and mortality in patients with severe liver failure. It is also an important factor in inborn errors of the urea cycle, Reye's syndrome, organic acidurias, valproate toxicity, transient hyperammonemia in infants, and idiopathic hyperammonemia.

The pathology of hyperammonemia, particularly HE, suggests that astrocytes play a crucial role in this condition (Norenberg, 1987). Astrocyte swelling represents the principal component of acute HE, while the presence of Alzheimer type II astrocytes is the main histological finding in chronic HE. No significant or consistent neu-

ronal changes have been identified (Norenberg, 1981). Because of the critical role of astrocytes in neurotransmission and CNS bioenergetics, we have proposed that astroglial dysfunction (gliopathy) and associated derangement in glial-neuronal interactions represent major aspects in the pathogenesis of ammonia neurotoxicity (Norenberg *et al.*, 1997).

Cerebral ammonia is chiefly metabolized to glutamine in astrocytes, due to predominant localization of glutamine synthetase in these cells (Norenberg and Martinez-Hernandez, 1979). Physiological levels of glutamine thus formed in astrocytes is released into the extracellular space and is taken up by neurons to generate glutamate and ammonia, a reaction mediated by phosphate-activated glutaminase (PAG). In addition, glutamine can also be metabolized to glutamate and ammonia in astrocytes, as evidenced by studies in culture (Kvamme *et al.*, 1992) as well as in vivo (Subbalakshmi and Murthy, 1985) showing that astrocytes possess PAG.

This article highlights the role of the mitochondrial permeability transition (MPT) as a major factor in the cellular dysfunction associated with ammonia neurotoxicity. The role of oxidative stress will be emphasized as a

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causal factor in the induction of the MPT. Mitochondrial dysfunction resulting from ammonia neurotoxicity as a consequence of the MPT will be discussed. Lastly, recent concepts on potential mechanism(s) of the ammonia-induced MPT will be presented.

CEREBRAL ENERGY METABOLIC FAILURE IN AMMONIA TOXICITY

The concept that ammonia disturbs cerebral energy metabolism has long been proposed (see Rama Rao and Norenberg, 2001 and references therein). Ammonia is known to interfere with various metabolic pathways of cerebral energy metabolism including inhibition of α -ketoglutarate dehydrogenase; stimulation of Na^+ , K^+ -ATPase resulting in depletion of ATP; impairment in the oxidation of pyruvate and glutamate; disturbance in the operation of the malate-aspartate shuttle; reduction in the state III mitochondrial respiration; and inhibition of the activity and expression of electron transport chain enzymes. Some of the abnormalities have been reproduced in cultured astrocytes exposed to pathophysiological concentrations of ammonia. In addition, several studies have shown morphologic changes in mitochondria in HE/hyperammonemia, principally swelling of the matrix and intracristal space (Gregorios *et al.*, 1985; Norenberg, 1977; Norenberg *et al.*, 2002).

OXIDATIVE STRESS IN AMMONIA TOXICITY

Oxidative stress is an evolving concept in HE and ammonia toxicity. Increased superoxide production and reduced activities of antioxidant enzymes have been reported in brains of rats subjected to acute ammonia toxicity (Kosenko *et al.*, 1997). Consistent with these findings, biphasic responses of total glutathione (GSH) were identified in cultured astrocytes exposed to 5 mM NH_4Cl . At early time points (up to 6 h) GSH levels were reduced by ammonia, whereas at later time points (up to 72 h), a progressive increase in GSH content occurred (Murthy *et al.*, 2000a,b). Lowered levels of GSH in astrocytes in early phase of ammonia exposure is consistent with the concept that ammonia induces oxidative stress in astrocytes. The later increase in GSH may represent an adaptive response to oxidative stress.

To examine the cellular basis of oxidative stress in ammonia toxicity, free radical production was measured employing the fluorescent probe 5-(and-6)-carboxy-2'-7'-dichlorofluorescein diacetate (DCFDA). These studies demonstrated that ammonia stimulated the production of free radicals in a dose-dependent manner. These data also disclosed that ROS levels remained elevated for at

least 4 h after exposure to ammonia. At the earliest time point (3 min) there was a robust increase in free radical production followed by a transient but significant reduction up to 2 h (but still higher than control); at 4 h the increase was similar to that observed at the 3 min time point (Murthy *et al.*, 2001; Rama Rao *et al.*, 2003a). This pattern of increase in ROS production by ammonia (2–4 h) is consistent with a concomitant decrease (up to 6 h) in astrocytic GSH levels as described above.

THE MITOCHONDRIAL PERMEABILITY TRANSITION

The potential involvement of mitochondrial dysfunction and oxidative stress in ammonia neurotoxicity prompted our investigation into the possible role of the mitochondrial permeability transition (MPT) in hyperammonemia. The MPT is characterized by a sudden increase in the permeability of the inner mitochondrial membrane to small molecules (<1500 Da). This is due to the opening of a specific permeability transition pore in the inner mitochondrial membrane, usually in response to an increase in mitochondrial Ca^{2+} levels. This leads to a collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$) that is created by the pumping out of protons by the electron transport chain. Loss of the $\Delta\Psi_m$ leads to colloid osmotic swelling of the mitochondrial matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis, and the generation of ROS. For reviews, see Zoratti and Szabo (1995) and Bernardi *et al.* (1998). The most specific blocker of the MPT is cyclosporin A (CsA), which competitively inhibits the mitochondrial matrix protein cyclophilin D from binding to pore domains (Crompton *et al.*, 1998).

To determine whether ammonia treatment of cultured astrocytes was associated with a change in the $\Delta\Psi_m$, a consequence of the MPT, astrocytes were treated with 5 mM NH_4Cl and examined for changes in the $\Delta\Psi_m$ using the potentiometric fluorescent dyes JC-1 and TMRE. Astrocytes exposed to ammonia showed a significant dissipation of the $\Delta\Psi_m$ in a time- and concentration-dependent manner. These studies also demonstrated that pretreatment with CsA (1–5 μM) blocked the ammonia-induced dissipation of the $\Delta\Psi_m$ (Bai *et al.*, 2001; Rama Rao *et al.*, 2003a) (Fig. 1), suggesting that ammonia was inducing the MPT.

To directly visualize permeability changes in mitochondria in situ, the calcein fluorescence method was employed (Petronilli *et al.*, 1999). Calcein/AM enters cells and becomes fluorescent upon de-esterification. Coloadings of cells with cobalt chloride quenches the fluorescence in the cell, except in mitochondria, since cobalt is

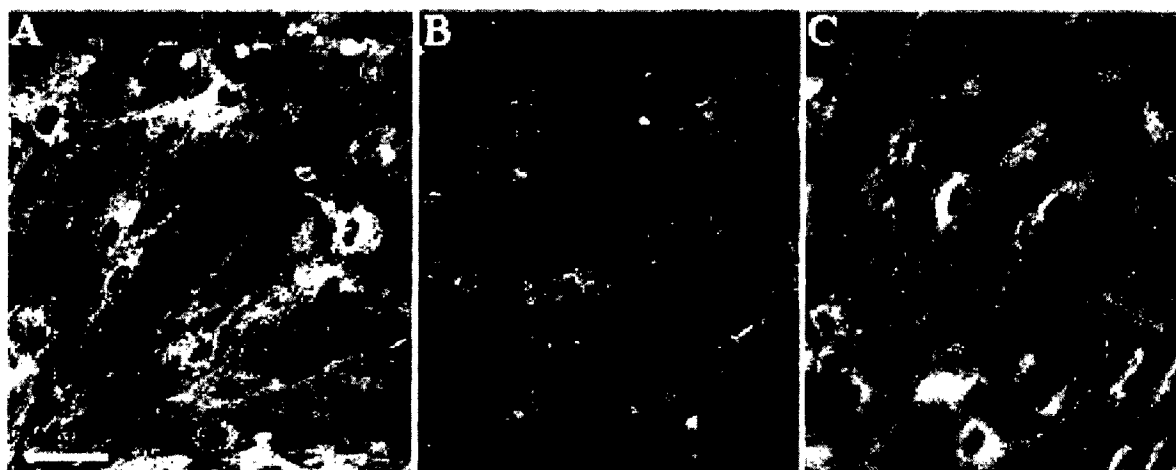


Fig. 1. Effect of 5 mM NH_4Cl on TMRE fluorescence in cultured astrocytes. Cells were loaded with 25 nM TMRE for 20 min. (A) Control astrocytes show prominent fluorescence. (B) Ammonia-treated astrocytes show decreased fluorescence. (C) Astrocytes treated with 1 μM CsA and ammonia is similar to control. Scale bar, 10 μm .

impermeable across mitochondrial membranes. However, during induction of the MPT, cobalt enters mitochondria and quenches the calcein fluorescence. Treatment of cultured astrocytes with ammonia (24 h) caused a significant reduction in the fluorescent intensity of calcein, which was significantly blocked by pretreatment with CsA (1 μM) (Fig. 2).

The ammonia-induced MPT in cultured astrocytes was significantly attenuated by various antioxidants, including SOD (25 U/mL), catalase (250 U/mL), desferroxamine (40 μM), *N*-*t*-butyl- α -phenyl-nitrone (PBN;

250 μM), supporting the notion that oxidative stress plays a major role in the ammonia-induced MPT in astrocytes (Jayakumar *et al.*, 2002).

ROLE OF GLUTAMINE IN THE MECHANISM OF AMMONIA NEUROTOXICITY

While ammonia is believed to be responsible for the neurological abnormalities associated with HE and other hyperammonemic syndromes, growing evidence



Fig. 2. Induction of the MPT in astrocytes by ammonia as demonstrated by calcein fluorescence. (A) Control astrocytes loaded with 1 μM calcein and quenched with cobalt show brightly stained mitochondria. (B) Astrocytes treated with ammonia (5 mM) for 24 h and then loaded with calcein show a significant loss of mitochondrial calcein fluorescence, consistent with the induction of the MPT. (C) Cotreatment with CsA (1 μM) prevents the loss of calcein fluorescence by ammonia. Scale bar, 10 μm .

supports the view that glutamine, a byproduct of ammonia metabolism, plays a major role in the deleterious effects of ammonia. Various abnormalities associated with ammonia toxicity such as seizures, depressed glucose utilization, altered CNS metabolism, vascular CO₂ responsiveness, edema, and astrocyte swelling can be blocked by administration of methionine sulfoxamine (MSO), an inhibitor of glutamine synthetase (Rama Rao *et al.*, 2003b and references therein).

Earlier studies showed that MSO completely blocked the effect of ammonia on the MPT (Bai *et al.*, 2001), as well as free radical production (Murthy *et al.*, 2001). These findings suggested that glutamine was mediating the effects of ammonia on the MPT and free radical formation. Subsequent studies have examined the role of glutamine directly. Cultured astrocytes treated with glutamine (4.5–7 mM for 24 h) caused a significant dissipation of $\Delta\Psi_m$ as well as decreased mitochondrial calcein fluorescence, both of which were completely blocked by CsA (Rama Rao *et al.*, 2003b). In addition, glutamine significantly increased free radical production in cultured astrocytes, which was also completely blocked by CsA (Jayakumar *et al.*, 2004).

To investigate the potential mechanism by which glutamine induces free radicals and the MPT, cultured astrocytes were treated with 6-diazo-5-oxo-L-norleucine (DON; 1 mM), an inhibitor of phosphate-activated glutaminase (PAG). DON completely blocked the glutamine-induced free radical production (Jayakumar *et al.*, 2004). Since essentially all of the glutamine is metabolized in mitochondria by PAG, high levels of ammonia will be generated in these organelles leading to the production of free radicals and the induction of the MPT. We envision glutamine acting as a “Trojan horse” by providing high levels of ammonia, leading to oxidative stress and mitochondrial dysfunction.

ASTROCYTIC MITOCHONDRIA ARE MORE VULNERABLE TO THE AMMONIA-INDUCED MPT

It is noteworthy that astrocytic rather than neuronal mitochondria are predominantly vulnerable to MPT induction by ammonia (Bai *et al.*, 2001). Similarly, glutamine had no effect on free radical production in cultured neurons (Jayakumar *et al.*, 2004). There are two possibilities to explain these findings. First, there is evidence of heterogeneity of mitochondria among neurons and astrocytes (Blokhuys and Veldstra, 1970), and it is possible that neuronal mitochondria may be more resistant to induction of the MPT by ammonia. Supporting this possibility,

Fiskum *et al.* (2000) demonstrated a greater resistance of neuronal mitochondria to the effects of Ca²⁺ overload and the subsequent induction of the MPT as compared with astrocytic mitochondria. Second, the selective vulnerability of astrocytes to the ammonia-induced MPT may be due to high levels of glutamine in astrocytes since ammonia is metabolized to glutamine in astrocytes but not in neurons.

ROLE OF THE MPT IN ASTROCYTE SWELLING

Astrocyte swelling represents a significant component of the brain edema in fulminant hepatic failure (FHF) (Córdoba and Blei, 1996). While the mechanism of edema associated with FHF is not completely understood, elevated ammonia levels have been strongly implicated in this disorder (Clemmesen *et al.*, 1999). Studies employing cultured astrocytes (Norenberg *et al.*, 1991) and brain slices (Ganz *et al.*, 1989) exposed to pathophysiological concentrations of ammonia have demonstrated prominent astrocyte swelling. More recently, ammonia has been shown to upregulate the water channel protein aquaporin4 (AQP4), suggesting that AQP4 may be responsible for astrocyte swelling (Rama Rao and Norenberg, 2003c). Collectively, there is compelling evidence that supports a major role of ammonia in the astrocyte swelling associated with hyperammonemia.

Since ammonia has been shown to induce the MPT and mitochondrial dysfunction, the role of the MPT on astrocyte swelling was assessed. Pretreatment of cultured astrocytes with different concentrations of CsA (0.1–1 μ M) significantly blocked the astrocyte swelling caused by ammonia. Parallel studies also demonstrated that CsA treatment significantly blocked the ammonia-mediated increase in AQP4 expression (Rama Rao and Norenberg, 2003d). Additionally, antioxidants significantly blocked the ammonia-induced astrocyte swelling (Murthy *et al.*, 2000). These studies support the role of the MPT and oxidative stress in the astrocyte swelling and brain edema associated with hyperammonemic states.

CONCLUDING REMARKS

In summary, ammonia induces the MPT in cultured astrocytes but not in cultured neurons, highlighting the critical role that astrocytes play in the toxic effects of ammonia. These effects of ammonia on the MPT were prevented by cyclosporin A. Ammonia-induced astrocyte swelling was blocked by CsA suggesting a major role of the MPT in this process. Our studies also suggest that

glutamine likely mediates the effect of ammonia in the induction of oxidative stress as well as the MPT. We propose that oxidative stress and the MPT represent key pathogenetic factors in ammonia neurotoxicity. These findings provide potential therapeutic targets for HE and other hyperammonemic states.

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The Mitochondrial Permeability Transition as a Target for Neuroprotection

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Mitochondria serve as checkpoints and amplifiers on cell death pathways. In the central nervous system, mitochondrial involvement seems essential for normal expression of cell death phenotypes, and interference with these pathways thus seems a reasonable approach to neuroprotection. We have been involved in examining the potential involvement of the mitochondrial permeability transition (mPT) as one of several possible mechanisms by which mitochondria may be drawn into these death cascades. This possibility, though still controversial, is supported by evidence that factors that may stimulate mPT induction are associated with some forms of cell death (e.g., in stroke) and are modulated by diseases of the central nervous system (e.g., Huntington's). Evidence of neuroprotection seen with compounds such as *N*-Met-Val cyclosporine also support this possibility.

KEY WORDS: Mitochondria; permeability transition; neuroprotection; apoptosis; oxidants; zinc; aldehydes; Huntington's disease.

INTRODUCTION

Recognition that mitochondria could release triggers of cell death, and that at least one of these triggers, cytochrome *c*, was sufficient for the latter stages of cell death, highlighted the potential gatekeeper role of mitochondria in cell death cascades. We focus our work in this area on the mitochondrial permeability transition (mPT), which has been defined primarily based on studies in purified liver and heart mitochondria. The term mPT refers to the opening of pores in the inner mitochondrial membrane that allows free diffusion of all solutes <1.5 kD. Therefore, mPT induction leads to loss of the proton gradient,

to inability to conduct oxidative phosphorylation, and to a potentially lethal efflux of mitochondrially sequestered calcium into the cytosol. mPT-like events have been observed in mitochondria isolated from CNS tissues (Kristal and Dubinsky, 1997), and mPT induction also has been experimentally associated with release of cytochrome *c*, AIF, and SMAC/DIABLO, which are the direct activators of the downstream cascades in both caspase-dependent and -independent cell death. Induction of an mPT has been linked to cytotoxicity following pathological insults, including stroke and excitotoxicity (Friberg and Wieloch, 2002). This said, the involvement of mPT in mitochondria from the CNS remains controversial, and available data suggest both PT-dependent and PT-independent events may be initiated by different pathogenic insults. Consistent with this, data from studies at the level of isolated mitochondria, cells, and intact animal models support the existence of "PT-like" events in the nervous system, but equally compelling data, suggest that "PT-like" events in the nervous system must be significantly different than those in the liver or heart, and that cell death in the CNS may often be "PT-independent" (Andreyev *et al.*, 1998; Andreyev and Fiskum, 1999; Berman *et al.*, 2000; Berman and Hastings, 1999; Kristal and Dubinsky, 1997; Martinou

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and Green, 2001). Here, we will focus on some of the pieces of evidence from our work that supports a role for PT in acute neurological injury and in chronic neurodegeneration.

BEYOND CALCIUM AND NON-SPECIFIC ROS—WHAT MODIFIES mPT SUSCEPTIBILITY?

One question about the relevance of mPT for cell death *in vivo* has followed from acknowledgement that the conditions used to study mPT *in vitro*, such as the lack of adenine nucleotides, may not translate well to *in vivo* circumstances. A second question concerns whether there may be disease-specific modifiers of mPT induction. We have been trying to address these issues by examining small molecules that may contribute to mPT induction in specific circumstances. Potential small molecule modulators of the mPT include, among others, Zn^{2+} , ganglioside GD3 (Kristal and Brown, 1999), the reactive aldehydes 4-hydroxyhexenal (HHE, a lipid peroxidation byproduct; Kristal *et al.*, 1996), 3,4-dihydroxyphenylacetaldehyde (DOPAL; Kristal *et al.*, 2001), peroxynitrite (Packer and Murphy, 1995; Brookes and Darley-Usmar, 2004), and arachidonic acid (Scorrano *et al.*, 2001). We have also examined whether changes in physiological status (e.g., Huntington's disease, impaired respiration) may be associated with changes in susceptibility to mPT induction.

Elevation of free intracellular Zn^{2+} has been observed in both heart and brain after ischemia/reperfusion or excitotoxic insults. Koh *et al.* (1996) found a one-to-one correspondence between neurons with elevated Zn^{2+} and markers of cell death following ischemia-reperfusion. Strikingly, in this study, neurons were protected from ischemia-reperfusion injury by chelators of Zn^{2+} but not Ca^{2+} , implicating Zn^{2+} in cell death. These and other studies implicate Zn^{2+} at the cellular and organism level, studies by ourselves and others implicate Zn^{2+} at the mitochondrial level as an mPT inducer and as an inhibitor of α -ketoglutarate dehydrogenase (Brown *et al.*, 2000). Induction of an mPT in the presence of Zn^{2+} shows both quantitative and qualitative similarities and differences relative to that involving Ca^{2+} . As two examples, effects of Zn^{2+} rapidly plateau and reverse, whereas those of Ca^{2+} do not. Zn^{2+} is also associated with a rapid, irreversible depolarization.

Monoamine-derived aldehydes have been suggested as possible toxicants in Parkinson's and Alzheimer's diseases. Consistent with a possible involvement of mPT, we had previously shown that hydroxyhexenal (HHE), a cytotoxic lipid peroxidation byproduct, accelerates mPT

induction at femtomolar concentrations (Kristal *et al.*, 1996). We have demonstrated that 3,4-dihydroxyphenylacetaldehyde (DOPAL), the direct MAO metabolite of dopamine, is more cytotoxic in neuronally differentiated PC12 cells than dopamine and several of its metabolites. DOPAL is also a potent mPT inducer (Kristal *et al.*, 2001). These data and others are consistent with a model in which DOPAL-induced mitochondrial damage, including induction of the mPT, contributes to disease progression in PD. Furthermore, DOPEGAL [3,4-dihydroxyphenylglycolaldehyde], the monoamine metabolite of epinephrine and norepinephrine, and a molecule suggested to contribute to Alzheimer's disease, is also a costimulators of the mPT.

Huntington's disease is a chronic progressive neurological disease that may also include systemic manifestations, including wasting. These aspects of the disease are modeled in several available transgenic mouse lines, including the R6/2 mouse. We have shown that the advanced stages of illness in this animal model are associated with an increased susceptibility to induction of the mPT in isolated liver mitochondria. This data has both similarities and differences from that previously presented by others (Panov *et al.*, 2002). Increased susceptibility was robustly observed under several different experimental conditions. Comparison with previous work on the mPT in diabetic rodents suggests that the effects observed are not a consequence of the diabetes that occurs in the R6/2 model. Increased susceptibility to mPT induction were independent of alterations in mitochondrial Ca^{2+} transport, endogenous Ca^{2+} load, respiration, or initial mitochondrial membrane potential ($\Delta\Psi$). Additional data obtained are consistent with the existence of a subpopulation of mitochondria that readily or constitutively exhibit the open conformation of the mPT pore *in vivo*. These data implicate further a systemic role for mutant huntingtin, and provide further evidence for a mitochondrial defect as a consequence of the gene mutation.

Activity of the tricarboxylic acid cycle component α -ketoglutarate dehydrogenase complex (KGDHC) is notably decreased in Alzheimer's and in several other neurodegenerative conditions. If this change is causally-linked to disease processes, then it is reasonable to expect that this linkage would be mediated by effects on mitochondrial physiology. In isolated rat forebrain nonsynaptosomal mitochondria, inhibition of KGDHC exerts coincident effects on $\Delta\Psi$, Ca^{2+} transport, and Ca^{2+} retention as well as ruthenium red insensitive, Ca^{2+} -mediated loss of mitochondrial membrane potential. The latter phenomenon is conceptually similar to the changes associated with mPT. In isolated liver mitochondria, a system more amenable to mechanistic evaluation, inhibition of KGDHC facilitated

mPT induction. This facilitation was independent of $\Delta\Psi$ during state 4 respiration, Ca^{2+} transport, and overall oxygen consumption. In contrast, progressive inhibition of respiration mediated by other substrates minimally affected or delayed mPT induction. These data suggest the potential for direct linkages between impaired KGDHC activity and neurodegenerative, in addition to cognitive, changes.

INTERVENTIONS

While this evidence is consistent with induction of an mPT during neurodegeneration and neurological changes, it remains unclear whether mPT is on the causative pathway of cell death, or whether it is simply a downstream effect related to overall cellular collapse, which includes, for example, oxidative damage to overall cellular collapse, which includes, for example, oxidative damage to components of the oxidative phosphorylation system. Although neuroprotection mediated by CsA was initially cited as evidence for causal involvement of mPT in ischemic injury, this is now appreciated to be problematic as CsA also affects calcineurin, the blockade of which itself has been shown to be neuroprotective. Similar "lack of specificity" arguments hold for other compounds, such as minocycline.

Minocycline is a second generation tetracycline antibiotic known to be protective in models of stroke, spinal cord injury, and neonatal hypoxia-reperfusion injury. While our recent work links minocycline to prevention of mPT-mediated release of mitochondrially-sequestered protein factors that facilitate both caspase-dependent and -independent cell death pathways, other actions of minocycline have been identified, and the use of minocycline to build a case for mPT involvement awaits a more mechanistic study of the actions of minocycline. In addition, while minocycline appears to prevent mPT-mediated release of cytochrome c, protection is highly atypical and displays unexpected properties, including an associated loss of mitochondrial membrane potential, an apparently stoichiometric response, and a sharp, biphasic dose response.

Arguably, the best direct test of the hypothesis that mPT lies on the causative pathway of clinically relevant cell death, at least in stroke, comes from the studies using *N*-Met-Val-CysA—a nonimmunosuppressive analog of CsA reputed not to interact with calcineurin. This compound reduces infarct size in a rat model of transient focal ischemia (Friberg and Wieloch, 2002). The universal acceptance of mPT involvement in stroke remains limited however, at least in part, because of the reliance on

data from a single drug, and the limited availability and characterization of its analog. Furthermore, even in stroke it appears that availability of CsA is limited by the blood-brain barrier. Thus, there is a need to show that other characterized agents can modulate mPT induction and protect against cerebral infarction, both to answer this central mechanistic question in the pathogenesis of stroke-related neuropathology and to help reduce its clinical effects.

Over the past 2 years, we were 1 of 30 projects re-examining FDA-approved drugs. The purpose of these screens was to identify previously unknown activities of FDA-approved compounds so that these drugs might be moved rapidly into clinical trials to treat previously unexpected conditions. Our assay examined the ability of such compounds to inhibit the mPT. Screening identified a subset of neuroactive medicines, including tricyclics and phenothiazines, as being protective against mPT induction (Stavrovskaya *et al.*, 2004). Indeed, some of these medications have been in clinical use since the 1950s, are known to cross the blood-brain barrier, and have been well-tolerated for long-term use, despite their side effects. Initial screens have identified drugs that appear protective at doses approaching those in clinical use. Literature searches reveal data that some of these compounds (e.g., desipramine, trifluoperazine) exert cytoprotective effects *in vitro* and protect against ischemia-reperfusion in some animal models, supporting the potential for these drugs to be protective against excitotoxic injury. Because the side effects of different tricyclics vary, yet the protection appears mediated via similar mechanisms, it may be possible to use combinations of multiple tricyclics to reduce side effects while strengthening protection.

In summary, the mPT remains a plausible candidate for therapeutic intervention in stroke and other problems of both acute neurotoxicity and chronic neurologic neurodegeneration. Disease specific conditions that facilitate mPT induction may exist.

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Effect of Overexpression of Protective Genes on Mitochondrial Function of Stressed Astrocytes

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Antiapoptotic members of the Bcl-2 family have been shown to reduce ischemic brain injury in vivo and in vitro. Understanding early changes in respiration are important in understanding the cells response to stress and the mechanisms of protection afforded by overexpression of protective genes. This mini-review summarizes current knowledge regarding early responses of astrocytes to ischemia-like stress and the effects of overexpression of protective Bcl-2 family genes on astrocyte mitochondrial function. Overexpression of Bcl-x_L improves mitochondrial respiratory function, normalizes mitochondrial membrane potential, and reduces production of free radicals early after the imposition of a stress in primary cultured murine astrocytes.

KEY WORDS: Astrocytes; Bcl-2; Bcl-x_L; cytochrome *c*; mitochondria; mitochondrial membrane potential; reactive oxygen species.

Mitochondria are central to both normal cell function and the regulation of cell death. Within the brain astrocytes are crucial for neuronal metabolic, antioxidant, and trophic support, as well as normal synaptic function. In the setting of stress, such as during cerebral ischemia, astrocyte dysfunction may compromise the ability of neurons to survive. Despite their central importance, the response of astrocyte mitochondria to stress has not been extensively studied. Limited data already suggest clear differences in the response of neuronal and astrocytic mitochondria to oxygen-glucose deprivation. Measurement of mitochondrial enzymatic activity by Almeida *et al.* (2002) demonstrated oxygen glucose deprivation (OGD) induced mitochondrial dysfunction in neurons at durations that left astrocyte activity intact. Loss of astrocyte mitochondrial membrane potential with OGD was not associated with irreversible injury, but required about 1 h to recover once oxygen and glucose were restored (Reichert *et al.*, 2001).

Prominent mitochondrial alterations during stress that can contribute to cell death include reduced ATP production, increased production of reactive oxygen species

(ROS), and release of death regulatory and signaling molecules from the intermembrane space. In response to stress mitochondrial respiratory function and membrane potential can also change, and these changes depend in part on cell type. Bcl-2 family proteins are the best-studied regulators of cell death, and mitochondria are a major site of action for these proteins (Gross *et al.*, 1999). The Bcl-2 family is divided into antiapoptotic members including Bcl-2 and Bcl-x_L and proapoptotic members such as BAX and BID (Adams and Cory, 1998; Tsujimoto, 1998; Tsujimoto and Shimizu, 2000a,b). Although much data supports the role of Bcl-2 family proteins in the regulation of some of these mitochondrial alterations, this remains an area of active investigation.

To better understand the ability of Bcl-x_L to protect astrocytes we examined mitochondrial function early after the imposition of oxidative stress. We previously reported that overexpression of Bcl-x_L increases astrocyte survival after both hydrogen peroxide exposure and glucose deprivation (GD) injury (Xu *et al.*, 1999a). Here we describe our recent findings on the effects of Bcl-x_L on accumulation of reactive oxygen species (ROS), cell respiration, and

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Key to abbreviations: GD, glucose deprivation; OGD, oxygen glucose deprivation; ROS, reactive oxygen species; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TMRE, tetramethylrhodamine ethylester.

Table I. Effect of Bcl-x_L Overexpression on Astrocyte Mitochondrial Membrane Potential With Glucose Deprivation

	Control, -G	Lac-Z, -G	Bcl-x _L , -G	Control, +G
0 h GD	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.01
3 h GD	1.25 ± 0.03*	1.26 ± 0.04*	0.98 ± 0.03 [#]	1.00 ± 0.01 [#]
5 h GD	0.84 ± 0.01*	0.80 ± 0.02*	0.96 ± 0.02 [#]	0.41 ± 0.01 ^a

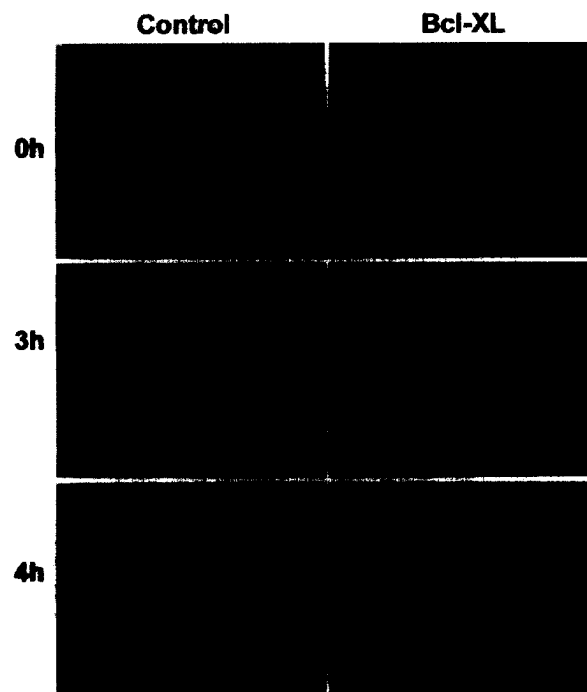
Note. TMRE mitochondrial fluorescence with glucose deprivation (-G) in uninfected, Lac-Z, and Bcl-x_L overexpressing astrocytes was normalized to the basal fluorescence for each cell at the start of the experiment. Values are means ± SD of at least 50 astrocytes per condition. [#] indicates $P < 0.05$ compared with the Lac-Z control at the same time, * indicates $P < 0.05$ compared with 0 h same condition by ANOVA followed by Scheffe's test.

^aCCCP (5 μM) was used to induce a collapse of mitochondrial membrane potential in control cultures not deprived of glucose (+G).

mitochondrial membrane potential in astrocytes exposed to hydrogen peroxide or GD. Overexpression of Bcl-x_L or a control gene was achieved in primary astrocyte cultures from mouse cortex using retroviral vectors (Xu *et al.*, 1999a). This resulted in cultures in which essentially all the cells express the gene of interest.

Tetramethylrhodamine ethylester (TMRE), a potentiometric fluorescent dye that incorporates into mitochondria was used at 100 nM to determine the time course of changes in mitochondrial membrane potential (Ouyang *et al.*, 2002). Distinct changes in astrocyte mitochondrial membrane potential were observed in response to H₂O₂ exposure as compared to GD. H₂O₂ induced a decrease in mitochondrial membrane potential (Ouyang *et al.*, 2002), while GD caused an initial increase (at about 3 h, Table I, Fig. 1) followed by a decrease (between 4 and 5 h) (Ouyang *et al.*, 2002). Although both injuries involve oxidative stress, peroxide exposure may more rapidly damage oxidation sensitive mitochondrial proteins. Both the transient increase in the mitochondrial membrane potential after 2–3 h of glucose deprivation, and subsequent decrease in mitochondrial membrane potential were prevented by Bcl-x_L overexpression (Fig. 1) (Ouyang *et al.*, 2002).

Oxygen consumption was measured in cortical astrocytes after increasing durations of GD (Ouyang and Giffard, 2003) using a modification of the method of Fiskum and colleagues (Fiskum *et al.*, 2000; Moreadith and Fiskum, 1984). Astrocytes were permeabilized by adding 0.01% digitonin and state III respiration was initiated by adding 0.4 mM ADP. Oligomycin (2 μg/mL) was used to initiate state IV respiration and the uncoupled rate was determined by addition of carbonylcyanide *m*-chlorophenylhydrazone (CCCP, 0.1 μM). State III respiration decreased significantly as early as 3 h after removal of glucose (Table II). At this time point state IV

**Fig. 1.** TMRE was used to image mitochondrial membrane potential in astrocytes subjected to GD. Pseudocolor images at 0 h (upper panel), 3 h (middle panel), and 5 h (lower panel) of control uninfected (left column) and Bcl-x_L overexpressing (right column) astrocytes are shown.

respiration and uncoupled respiration did not change. After 5 h of GD state IV respiration increased significantly and state III respiration declined further. In contrast, uncoupled respiration did not change much compared with 0 h GD. Although overexpression of Bcl-x_L did not change basal respiratory rates (Ouyang and Giffard, 2003), when astrocytes were stressed, Bcl-x_L overexpression prevented

Table II. Mitochondrial Respiration of Astrocytes During Glucose Deprivation

	State III	State IV	Uncoupled
<i>Lac-Z</i>			
0 h GD	25.2 ± 3.1	5.1 ± 0.4	35.9 ± 3.6
3 h GD	18.6 ± 2.3*	4.9 ± 0.3	34.8 ± 4.2
5 h GD	16.4 ± 1.2*	8.1 ± 0.5*	31.1 ± 3.3
<i>Bcl-X_L</i>			
0 h GD	27.8 ± 2.5	5.5 ± 0.3	38.9 ± 2.5
3 h GD	29.9 ± 1.6 [#]	5.7 ± 0.3 [#]	40.4 ± 2.6
5 h GD	28.3 ± 2.3 [#]	6.9 ± 0.4 [#]	35.7 ± 2.1

Note. Values are means ± SD nmol O₂/min/mg protein. [#] indicates $P < 0.05$ compared with the Lac-Z control at the same time, * indicates $P < 0.05$ compared with 0 h same condition by ANOVA followed by Scheffe's test.

the decrease in state III respiration and moderated the increase in state IV respiration (Table II).

Using the ROS-sensitive fluorescent dye hydroethidine, we recently demonstrated that with GD or H₂O₂, cultured astrocytes showed immediate and rapid increases in ROS accumulation which were markedly reduced by overexpressing Bcl-x_L (Ouyang *et al.*, 2002). We previously found that peroxide exposure was associated with an increase in intracellular calcium as measured with Fura-2 (Ouyang *et al.*, 2002). However, the improved survival with Bcl-x_L overexpression at 400 μ M peroxide did not correlate with a reduction in the increase in intracellular calcium observed (Ouyang *et al.*, 2002).

Fluorescence immunocytochemistry was performed to detect cytochrome *c* release (Ouyang and Giffard, 2003). At 3 h GD cytochrome *c* is localized in mitochondria while at 5 h about 1/4 of control astrocytes showed an evenly distributed immunostaining pattern demonstrating release of cytochrome *c* from the mitochondria to the cytosol. Mitochondrial morphology changed from elongated to punctate in those cells. We observed that Bcl-x_L overexpression prevented loss of cytochrome *c* from mitochondria and the change in mitochondrial morphology (Ouyang and Giffard, 2003). We assessed cell death at these times by staining with Hoechst dye 33258 and propidium iodide. While essentially no cells stained with propidium iodide at the beginning of the experiment, at 3 h of GD $4.2 \pm 0.1\%$ and after 5 h GD $15.4 \pm 0.4\%$ of the control cells stained with propidium iodide. This suggests that the control-injured cells releasing cytochrome *c* are dead or dying. GD induced astrocyte death is more rapid in cells being imaged than in sister cultures maintained in the dark.

We conclude that in addition to the well-established ability of the antiapoptotic Bcl-2 family members to block release of apoptotic factors from mitochondria, Bcl-x_L also improves mitochondrial respiratory function, normalizes membrane potential, and reduces production of free radicals by astrocytes subjected to oxidative stress. These improvements in astrocyte mitochondrial function may in part explain the ability of Bcl-x_L overexpressing astrocytes to protect wild type neurons co-cultured with them against GD and OGD (Xu *et al.*, 1999b).

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Astrocyte Mitochondria in In Vitro Models of Ischemia

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There is growing evidence that preservation of mitochondrial respiratory function during cerebral ischemia–reperfusion predicts the ultimate extent of tissue injury. Because neurons are selectively vulnerable to ischemic injury, many studies have focused on neuronal mitochondrial dysfunction in ischemia. However, positron emission tomography (PET) studies in animals and humans suggest that non-neuronal cells such as astrocytes may also experience mitochondrial metabolic compromise that contributes to ischemic necrosis. Astrocytes carry out a number of functions that are critical to normal nervous system function, including uptake of neurotransmitters, regulation of pH and ion concentrations, and metabolic support of neurons. Mitochondria are important for many of these actions. We have used a cell culture model of stroke, oxygen–glucose deprivation (OGD), to study the response of astrocyte mitochondria to ischemia, and to evaluate how changes in astrocyte mitochondrial function might affect neuronal survival and recovery after ischemia.

KEY WORDS: Tetramethylrhodamine ethyl ester; cytochrome c; mitochondrial permeability transition pore; nitric oxide synthase; cyclosporin A; confocal microscopy; cortical cell cultures; brain slice.

INTRODUCTION

Over the past decade, the central role that mitochondria play in diseases of the nervous system has become increasingly clear (Beal and Al, 1992; Fiskum, 2000; Wallace, 1999). Mitochondrial functional abnormalities have been linked to genetic neurological disorders and neurodegenerative diseases (Wallace, 1999). Mitochondrial dysfunction has also been implicated in ischemic injury, in which inadequate delivery of oxygen and glucose limits mitochondrial respiration. Recent studies suggest that, in fact, the degree of mitochondrial dysfunction in cerebral ischemia may be a critical determinant of the final extent of tissue injury.

Studies on mitochondria isolated from an ischemic brain suggest that ischemia–reperfusion can cause short- and long-term alterations in mitochondrial function (Hillered *et al.*, 1984; Schutz *et al.*, 1973; Sims *et al.*, 1986; Sims, 1991). After brief focal ischemia, iso-

lated mitochondria demonstrate defects in State 3 (ADP-dependent) and State 4 (ADP-independent) respiration. These deficits resolve after a brief period of reperfusion. Longer periods of ischemia lead to prolonged impairment in State 3 respiration (Sims, 1991). In addition, although mitochondrial metabolism normalizes soon after ischemia–reperfusion, many studies have found a secondary decline minutes to hours later (Rehncrona *et al.*, 1979; Sims and Pulsinelli, 1987; Zaidan and Sims, 1994). The cause of this delayed decline is proposed to be oxidative damage to mitochondria during reperfusion (Feng *et al.*, 1998; Sims and Pulsinelli, 1987), or the effect of fatty acids released during ischemia on mitochondrial function (Hillered and Chan, 1988; Sun and Gilboe, 1994). Decreased pyruvate dehydrogenase (PDH) activity is also impaired after ischemia–reperfusion (Cardell *et al.*, 1989; Zaidan and Sims, 1993).

The selective vulnerability of neurons to ischemic injury has been taken as an indication that neurons experience greater metabolic deterioration than astrocytes, which are relatively resistant to ischemia injury. Astrocytes also contain glycogen stores, which are presumed to allow them to maintain ATP production through glycolysis and mitochondrial membrane potential by reversal of the F_0F_1 -ATPase. Although loss of mitochondrial membrane

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potential has been documented in neurons exposed to ischemic conditions, as will be discussed, astrocytes also exhibit early mitochondrial depolarization when exposed to oxygen–glucose deprivation (OGD).

ASTROCYTE MITOCHONDRIAL DEPOLARIZATION DURING OGD

The in vitro oxygen–glucose deprivation model used for our studies has previously been described in detail (Goldberg and Choi, 1993; Bruno *et al.*, 1994). In this model, mixed cortical astrocytic–neuronal cocultures are exposed to oxygen–glucose deprivation (OGD) for 30–60 min. Fifty percent of neurons are irreversibly injured after 45 min of OGD, with 100% neuronal death produced by 60–70 min of OGD. In contrast, astrocytes in the same cultures are resistant to OGD-induced death, requiring 4 h for irreversible injury. Previous work with this model has found that even after 60 min of OGD, ATP levels are still at 70% of control levels. In addition, benzodiazepines and barbiturates worsen neuronal cell death through effects on mitochondrial function, reproducing findings in stroke patients receiving these two classes of agents, who have a worse prognosis, and suggesting that this in vitro model mimics many of the metabolic aspects of cerebral ischemia.

Using this system, we found that exposure to 45–50 min of OGD produced a 70% decrease in mitochondrial membrane potential (ψ_m) in astrocytes, determined by confocal imaging of tetramethylrhodamine ethyl ester fluorescence (Reichert *et al.*, 2001). This time point correlates with the previously reported rise in extracellular glutamate and with the onset of irreversible injury to neurons observed in this model (Goldberg and Choi, 1993). In our experiments, treatment with a nitric oxide synthase inhibitor (G N-Arg) partially blocked the decline in mitochondrial membrane potential (ψ_m), suggesting that nitric oxide (NO) or peroxynitrite were involved in loss of ψ_m . Brown and Borutaite (1999) have previously found that both NO and peroxynitrite can inhibit mitochondrial electron transport chain activity at multiple sites. We were unable to differentiate between a role for NO vs. peroxynitrite in our model, but given the low O_2 (0.2%) in the anaerobic chamber used for these experiments, it is unlikely that an extensive amount of peroxynitrite would be present.

Astrocyte ψ_m was also preserved during OGD by treatment with cyclosporine A, indicating that opening of the mitochondrial permeability transition pore (mtPTP) is involved in the loss of astrocyte ψ_m . Assembly of the mtPTP is triggered by several stimuli, including fatty acids, accumulation of mitochondrial calcium, and oxidative stress—events that are reported to occur during ischemia–reperfusion injury.

In the absence of OGD, astrocytes are clearly capable of using ATP to maintain ψ_m , by reversal of the F_0F_1 -ATPase to support ψ_m , but why astrocytes exposed to OGD fail to support ψ_m through this mechanism is not clear. Involvement of glutamate-receptor-mediated calcium entry and direct uncoupling by Ca^{2+} are unlikely because blocking AMPA/kainate receptors failed to protect astrocyte ψ_m (Reichert *et al.*, 2001). Coincubation with 1% bovine serum albumin (BSA, fatty acid free) also failed to modify ψ_m loss (Reichert and Dugan, unpublished), suggesting that fatty acid release from neurons is also not likely to be involved. Loss of ψ_m may involve glutamate, acting through the transporter, or adenine nucleotides released by neurons (Fig. 2). This may add to the growing body of literature describing extensive communication between neurons and astrocytes (Giaume and McCarthy, 1996; Kimelberg and Norenberg, 1989; Magistretti *et al.*, 1993).

Recovery of astrocyte ψ_m after reintroduction of O_2 and glucose was a gradual process, requiring >1 h. There were also ultrastructural changes in mitochondrial exposed to OGD, that persisted for a relatively prolonged period of time suggesting that OGD causes specific but reversible changes in astrocyte mitochondrial physiology beyond lack of O_2 and glucose (Fig. 1). CsA decreased the extent of morphological changes. It is possible that these changes may correspond to the mitochondrial swelling and matrix alterations reported in early post-ischemic brain using electron microscopy (Petito and Babiak, 1982).

IMPLICATIONS OF ASTROCYTE MITOCHONDRIAL DYSFUNCTION

There are a number of potential downstream effects of mitochondrial depolarization. In most cell types, such prolonged loss of ψ_m would activate apoptotic pathways and result in cell death. Depolarization might also lead to loss of intramitochondrial contents, such as ADP, leading to prolonged impairment in mitochondrial respiration and ATP production. If depolarization altered the production of reactive oxygen species, such as H_2O_2 , this might result in detrimental changes in intracellular and intercellular signaling through redox-sensitive pathways, such as Ras, Erk1/2, and NF κ B. We have been exploring each of these potential outcomes in turn (Fig. 2).

Initiation of the Apoptotic Cascade

Activation of the mtPTP can lead to mitochondrial depolarization and is associated in many cell types with release of cytochrome c from the inner mitochondrial

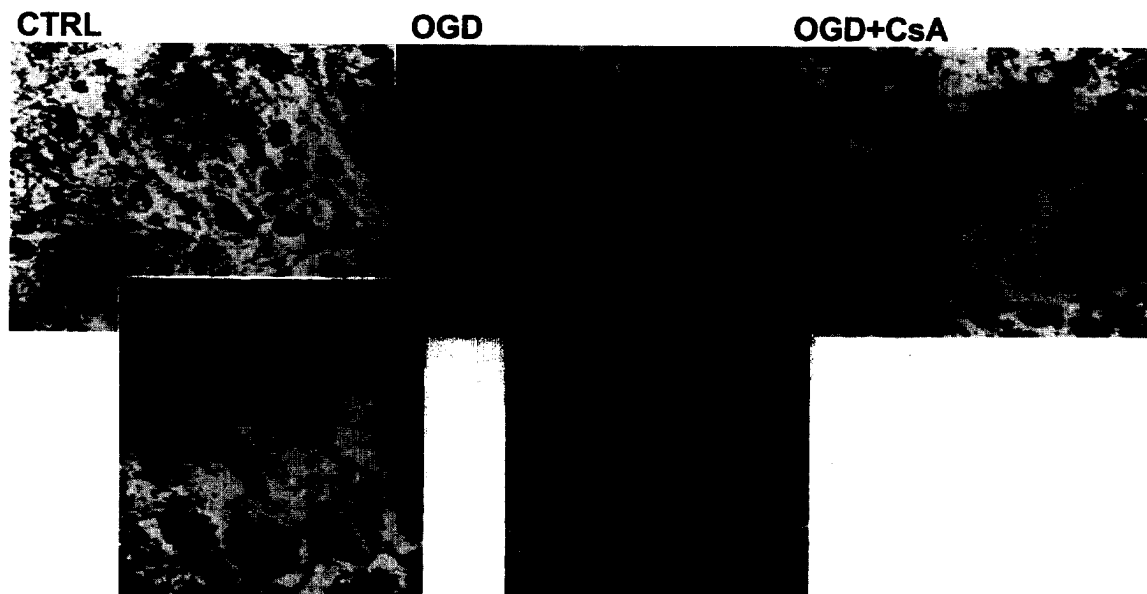


Fig. 1. Fluorescence confocal images of astrocyte mitochondria after OGD. Mixed cortical cultures were loaded with TMRE (50 nM) and exposed to 50 min OGD. Panels show control, OGD, and OGD plus cyclosporine A (10 μ M) conditions. Insets are at 4X magnification.

membrane (Halestrap *et al.*, 2000). The free cytochrome c may then activate caspase 9 through binding to apaf-1. Caspase 9, which resides in the mitochondrial matrix (as a pro-caspase), may be released when the barrier function of the inner membrane is lost, and can activate and work synergistically with caspase 3 to trigger downstream effectors of apoptosis (Thornberry and Lazebnik, 1998).

We observed a 20% loss of cytochrome c from mitochondria at the end of OGD that was partly blocked by CsA. However, release of cytochrome c was not accompanied by activation of either caspase-9 or caspase-3, suggesting that cytochrome c was blocked from activating the mitochondrial caspase cascade.

Decreased Energy Production and Altered Ion Homeostasis

In addition to its role in apoptosis, release of cytochrome c from mitochondria could alter mitochondrial function by hindering the efficient transfer of electrons through cytochrome aa_3 in cytochrome oxidase, enhancing upstream superoxide radical production from ubiquinone redox cycling (Wallace, 1999). Elimination of ψ_m abolishes mitochondrial Ca^{2+} uptake, and may impair many other aspects of mitochondrial metabolism, in addition to the most well known impairment of ATP production. Extensive data indicate that astrocytes are involved in a number of processes that affect neuronal survival, such as glutamate uptake, maintenance of extracellular pH and potassium, Ca^{2+} buffering, and transfer of lactate and/or

pyruvate to neurons as energy substrates (Anderson and Swanson, 2000; Forsyth, 1996; Magistretti *et al.*, 1993; Vernadakis, 1996; Walz, 2000). A number of these functions are dependent on mitochondrial membrane potential, and have been reported to be impaired early in ischemia (Benveniste *et al.*, 1984; Montgomery, 1994; Juurlink, 1997).

Altered ROS Production and Effects on Signaling

One additional effect of mitochondrial depolarization could be altered mitochondrial ROS generation. Whether ROS production would be enhanced or decreased by OGD–reperfusion is not entirely clear, and might vary with the duration of OGD, and the time after reperfusion. Mitochondria might be one source, but NADPH-depleted NOS might also contribute. Further work on the source(s) and timing of astrocyte ROS production during OGD is ongoing.

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radical (Dalton *et al.*, 1999; Finkel, 1999), nitric oxide, and peroxynitrite (Li *et al.*, 1998), act as signaling molecules to regulate kinase cascades, transporters, ion channels, and transcription factors. Our recent studies indicate that OGD alters activity of several signaling pathways, including MAPK (Fig. 2). To what extent changes in MAPK activity alter astrocyte and neuronal gene expression and function is an area of continuing investigation.

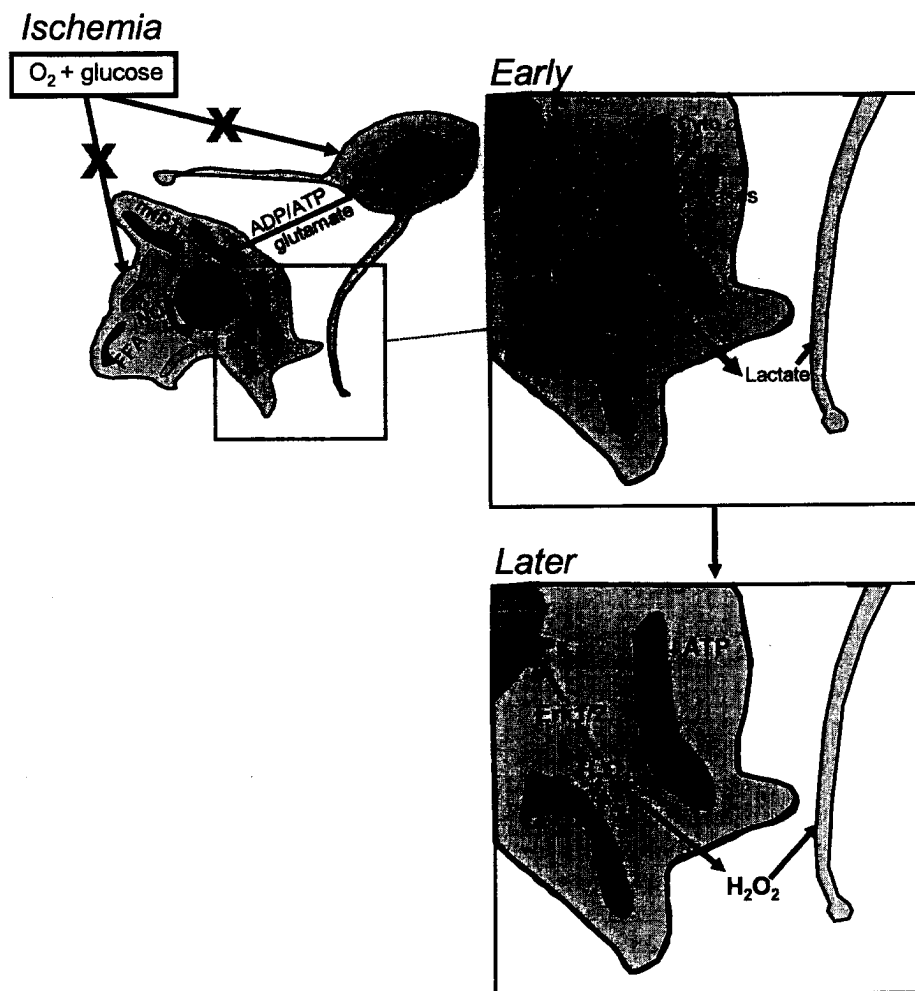


Fig. 2. Proposed sequence of astrocyte mitochondrial changes and secondary effects after OGD. (NO: nitric oxide; FFA: free fatty acid; mtPTP: mitochondrial permeability transition pore; MMP: metalloprotease) Decreased availability of O_2 and glucose is sensed by neurons and astrocytes. Astrocyte mitochondria depolarize, a process accelerated by a soluble factor from neurons (Kim-Han, unpublished), which might be glutamate through the transporter (Liao and Chen, 2003), or adenine nucleotides. Loss of ψ_m involves NO, the mtPTP (Reichert *et al.*, 2001), and possibly fatty acids (Chan *et al.*, 1988). ATP levels are preserved, but ATP is not used to support ψ_m , presumably due to blockage at the F_0F_1 ATPase. This could allow shunting of lactate to metabolically compromised neurons. Cytochrome c is released but fails to activate caspases. The ratio of cytochrome c to inhibitor of apoptosis proteins (IAP)—which appears to be lower in astrocytes—might be partially responsible (Dugan and Kim-Han, unpublished). At later time points, levels of H_2O_2 in astrocytes increase, modifying redox-dependent signaling pathways, such as Erk1/2. Potential sources include the ETC or NADPH-depleted NOS. Low levels of H_2O_2 might also be released from astrocytes to alter redox-sensitive systems in neurons.

SUMMARY

Depolarization of astrocyte mitochondria during OGD might have both beneficial and harmful effects on neuronal survival. Short-term loss of ψ_m would allow astrocytes to temporarily shift use of glycogen and glucose away from aerobic metabolism to glycolysis, increasing the amount of lactate available for delivery to metabolically impaired neurons (Fig. 2). This might be tolerated for as long as astrocyte glycogen stores were available. However, prolonged loss of ψ_m in astrocytes might be expected

to have injury-promoting effects during CNS ischemia. Astrocytes are involved in the normal maintenance of brain homeostasis, including several energy-dependent functions necessary for normal neuronal activity, e.g., regulation of extracellular K^+ , pH, and osmolality; export of metabolic intermediates; and rapid uptake of neurotransmitters (Kimelberg and Norenberg, 1989; Magistretti *et al.*, 1993; Walz, 2000). The ability of astrocytes to maintain these functions may, in fact, be a critical determinant of neuronal survival after ischemia (Aschner *et al.*, 1999; Juurlink, 1997; Marri and Juurlink, 1999;

Stanimirovic *et al.*, 1997). Furthermore, metabolic imaging studies have suggested that mitochondrial function in post-ischemic brain may be impaired for hours to days after the ischemic insult (Heiss *et al.*, 1997; Hoehn-Berlage, 1995; Ogasawara *et al.*, 2001; Takamatsu *et al.*, 2000; Wardlaw *et al.*, 1998). Loss of ψ_m and eventual energy failure in astrocytes might lead to an inability to provide these critical support functions during ischemia, thus exacerbating ischemic injury to neurons. Our data suggest that therapies targeted at astrocyte mitochondria might act synergistically with neuron-based strategies to provide protection to the ischemic brain.

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Neuroprotective Effects of Ischemic Preconditioning in Brain Mitochondria Following Cerebral Ischemia

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Numerous studies support the hypothesis that reperfusion following cerebral ischemia contributes substantially to ischemic injury and that mitochondrial dysfunction plays a central role. Defining the mechanisms by which mitochondrial dysfunction occurs may be important for the development of new therapies against delayed neuronal cell death. Ischemic preconditioning (IP) increases an organ's resistance to ischemic injury. There are two windows for IPC, one that requires several hours to develop and another one with a rapid setting (rapid window). However, the rapid window only provides neuroprotection for few days. We have recently determined that this lack of chronic protection by the rapid window was due to lack of protection against mitochondrial dysfunction.

KEY WORDS: Cerebral ischemia; bioenergetics; metabolism; anoxia.

The mechanisms leading to neuronal cell death after cerebral ischemia are complex. A well established fact in this field is that cells continue to die over months after a stroke, a phenomena that has been termed delayed cell death. Although not clearly defined, neuronal cell death may result from either apoptosis, necrosis, or a cell death mechanisms that is a mixture of these processes (Liou *et al.*, 2003; Martin *et al.*, 1998).

Numerous studies support the hypothesis that reperfusion following cerebral ischemia contributes substantially to ischemic injury (Chan, 1994; Choi, 1993; Siesjo and Smith, 1991; Watson and Ginsberg, 1989) and that mitochondrial dysfunction plays a central role (Abe *et al.*, 1995; Ankarcrona *et al.*, 1995; Fiskum *et al.*, 1999; Friberg and Wieloch, 2002; Schinder *et al.*, 1996; White and Reynolds, 1996, 1997).

Evidence of mitochondrial dysfunction following cerebral ischemia was described in previous studies, as a prominent change in redox activity of mitochondrial respiratory chain components in postischemic brain (Perez-Pinzon *et al.*, 1997a,b; Rosenthal *et al.*, 1995, 1997; Welsh

et al., 1982, 1991). This hyperoxidation of electron carriers is indicative of either a response to decreased substrate availability (Rosenthal *et al.*, 1995) and/or a reaction of mitochondrial complexes to reactive oxygen species (ROS) (Perez-Pinzon *et al.*, 1997b). Postischemic mitochondrial may also be a major source of ROS, and free radical-mediated damage has been linked to reperfusion injury following brain ischemia (Flamm *et al.*, 1978; Fridovich, 1979; Hall and Braughler, 1993; Kontos, 1989; Siesjo *et al.*, 1985; Vlessis *et al.*, 1990). However, recent findings suggest that this hyperoxidation may result from loss of electron carriers from mitochondria following cerebral ischemia, such as cytochrome *c* and NADH (Perez-Pinzon *et al.*, 1999c). The loss of cytochrome *c* from mitochondria might affect respiratory chain activity and/or it may trigger the apoptotic cascade (Charriaut-Marlangue *et al.*, 1996; Nitatori *et al.*, 1995). This is suggested by findings that apoptosis (programmed cell death) may be linked to mitochondria and their release of cytochrome *c* (Ankarcrona *et al.*, 1995; Kluck *et al.*, 1997; Schinder *et al.*, 1996; Yang *et al.*, 1997).

Additional evidence of mitochondrial dysfunction was described in studies from isolated brain mitochondria. Mitochondria isolated from ischemic brain exhibited decreases in state 3 respiratory rates of approximately 70% with NAD-linked respiratory substrates (Sciamanna *et al.*, 1992). Cafe *et al.* (1994) showed that nonsynaptosomal

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mitochondria were insensitive to ischemia but that they became dysfunctional in the late reperfusion phase. Mitochondria from synaptic terminals were greatly affected by ischemia but partially recovered during reperfusion. Sims and Pulsinelli (1987) also reported that in rat a model of forebrain transient ischemia the rate of oxygen consumption decreased in the CA1, CA3, and CA4 regions in the late reperfusion phase. This study was performed in homogenates from different brain subregions.

ISCHEMIC PRECONDITIONING

Ischemic preconditioning (IP) refers to the ability of a brief ("sublethal") ischemic episode, followed by a period of reperfusion, to increase an organ's resistance to injury (ischemic tolerance) following a subsequent ischemic event. This induction of tolerance against ischemia resulting from sublethal ischemic or anoxic insults has gained attention as a robust neuroprotective mechanism against conditions of stress such as anoxia/ischemia in heart and brain (Alkhulaifi *et al.*, 1993; Kato *et al.*, 1992; Kitagawa *et al.*, 1990; Lin *et al.*, 1992, 1993; Murry *et al.* 1986; Walker *et al.*, 1993). There are different preconditioning paradigms both in heart and brain. Among variations in preconditioning paradigms include, the number of pre-

conditioning insults, types of preconditioning insults, and time between preconditioning and the test insults.

In the past, most preconditioning studies in brain have suggested that several hours are required to develop the tolerant state. However, in recent studies we and others suggested that preconditioning with a rapid onset time course, similar to that in heart (within 1 h), can protect synaptic activity after anoxia in brain slices (Centeno *et al.*, 1999; Perez-Pinzon *et al.*, 1996, 1999a; Perez-Pinzon and Born, 1999; Schurr *et al.*, 1986; Schurr and Rigor, 1987) and reduce histopathology after ischemia in intact brain (Atochin *et al.*, 2003; Nakamura *et al.*, 2002; Perez-Pinzon, 2000; Perez-Pinzon *et al.*, 1997c; Stagliano *et al.*, 1999).

Thus, two windows have been identified (see Fig. 1), one that occurs very rapidly (within 1 h) (Perez-Pinzon, 2000; Perez-Pinzon *et al.*, 1997c; Stagliano *et al.*, 1999) and a second one that develops slowly (over days) (Kitagawa *et al.*, 1990). A difference between these two windows is that neuroprotection in the first window is transient. We observed that significant neuroprotection against histopathology was evident after 3 days of reperfusion, but after 7 days, histopathology was similar between controls and preconditioned rats (Perez-Pinzon *et al.*, 1997c). In contrast, when rats were preconditioned days prior to the "test" ischemic insult, the neuroprotection was robust

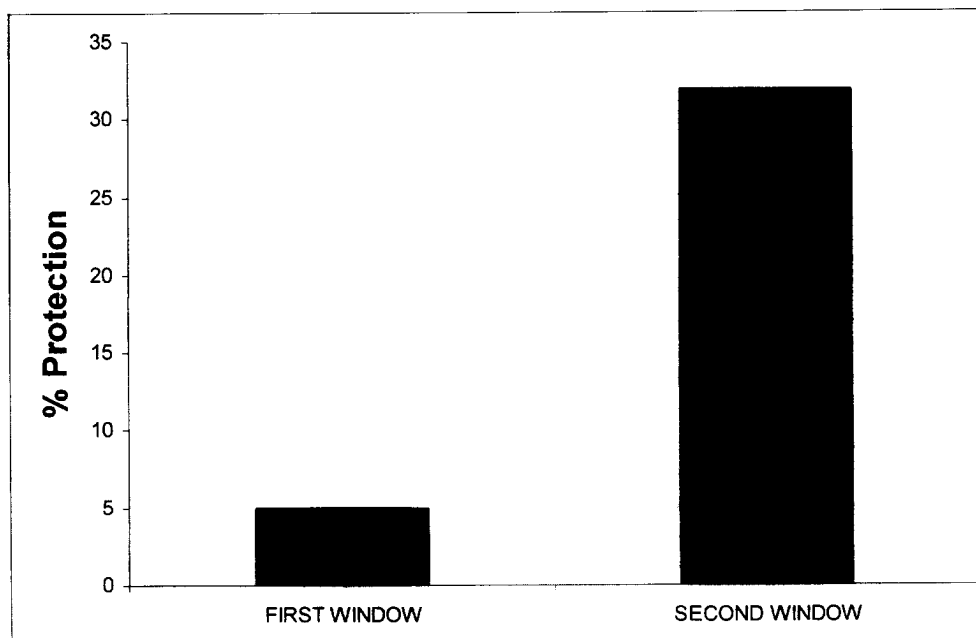


Fig. 1. Comparison between the % protection in the two windows of ischemic preconditioning. In the first window, IPC preceded the test ischemic insult by 30 min. In this window, IPC protected against histopathology at 3 days, but not 7 days of reperfusion. In contrast, when IPC preceded the test ischemic insult by 48 h, there was approximately 33% protection of normal neurons after 7 days of reperfusion.

and long lasting (Kitagawa *et al.*, 1990; Kurkinen *et al.*, 2001).

IPC and Mitochondrial Protection in the First Window

Recent studies from this laboratory and others suggest that preconditioning from the first window can protect ischemic injury, as assessed by the release of lactic dehydrogenase (LDH) or by synaptic activity recovery after the test insults in cell cultures (Reshef *et al.*, 1996), brain slices (Centeno *et al.*, 1999; Perez-Pinzon and Born, 1999; Perez-Pinzon *et al.*, 1996, 1999a; Schurr *et al.*, 1986; Schurr and Rigor, 1987) and reduce histopathology after ischemia in intact brain (Atochin *et al.*, 2003; Nakamura *et al.*, 2002; Perez-Pinzon, 2000; Perez-Pinzon *et al.*, 1997c, 1999b). However, protection only ensued if reperfusion was allowed to occur for 3 days but not beyond (Perez-Pinzon *et al.*, 1997c). Thus, it was important to ascertain whether this lack of chronic protection was due to less protection against mitochondrial dysfunction by IPC.

Indeed, we found that the first window of IPC could not protect mitochondria against the deficits in respiration through complexes I–IV (Perez-Pinzon *et al.*, 2002). Rates of respiration in presence of pyruvate + malate and succinate + glycerol – 3 – phosphate decreased in both the ischemia and in IPC groups marginally. However, significant decreases in the rate of respiration for complex IV were observed in both the ischemia and IPC groups. This decrease in the rate of respiration in the presence of complex IV substrates is suggestive of impairment in oxidative phosphorylation. This decrease in the rate of respiration at the level of complex IV was not accompanied by significant decreases in complex IV activity. A possible explanation for the decrease of respiration in complex IV and no change in complex IV activity in the ischemia group is that cytochrome *c* may be released from mitochondria following test ischemia. This contention is supported by previous studies. We found that the apparent mitochondrial hyperoxidation linked to brain dysfunction may be caused by disruption of the mitochondrial membrane and the concomitant loss of the mitochondrial electron carriers (Perez-Pinzon *et al.*, 1999c). In that study, cytosolic cytochrome *c* was increased following global cerebral ischemia and 30 min of reperfusion (same model of cerebral ischemia as in the current study); and conversely, reducible cytochrome *c* (presumably the intramitochondrial fraction of this cytochrome) was decreased following anoxia in hippocampal slices. This latter finding was correlated with NADH hyperoxidation that occurs following anoxia

in hippocampal slices and the first window of preconditioning was unable to protect against such hyperoxidation (Centeno *et al.*, 1999).

IPC and Mitochondrial Protection in the 2nd Window

In contrast to the lack of mitochondrial protection by IPC in the first window, IPC in the second window significantly protected mitochondria against the deficits in respiration through complexes I–IV (Dave *et al.*, 2001). As described above, many studies have demonstrated that reactive oxygen species (ROS) and the resulting oxidative stress play a pivotal role in neuronal cell death (Flamm *et al.*, 1978; Fridovich, 1979; Hall and Braughler, 1993; Kontos, 1989; Siesjo *et al.*, 1985; Vlessis *et al.*, 1990). There are two major regions in the electron transport chain where ROS are produced. One is complex I and the other complex III (Chance and Williams, 1956). Since oxidative stress is implicated in the pathophysiology that ensues after cerebral and cardiac ischemia (Kannan and Jain, 2000), one can surmise that a key mechanism by which IPC in the second window protects hippocampus against delayed neuronal cell death is by protecting mitochondrial oxidative phosphorylation.

However, the precise mechanism by which IPC affords protection to mitochondria remains undefined. A possible mechanism may involve upregulation of neuroprotective genes. Cai and Storey (1996) found that anoxia stress induces upregulation of the genes for NADH-ubiquinone oxidoreductase subunit (encoded by mitochondrial gene) and cytochrome oxidase subunit 1 (encoded by mitochondrial gene) in the anoxic-resistant turtle heart (Cai and Storey, 1996). We presume that IPC may upregulate certain genes responsible for the activities of these complexes, which may render hippocampal mitochondria resistant to “lethal” ischemia.

Another possible mechanism may involve better maintenance of ATP. There is considerable evidence for the role of ATP depletion, which follows hypoxic/ischemic insults, in the development of mitochondrial damage and the subsequent activation of downstream cell death pathways (Galeffi *et al.*, 2000). Yabe *et al.* (1997) have shown that in preconditioned heart, the glycolytic ATP production increases. They have also demonstrated that ATP and creatine phosphate concentration remained higher in preconditioned group as compared to nonpreconditioned group (Galeffi *et al.*, 2000). A higher level of ATP in preconditioned group may prevent cytochrome *c* release from the mitochondria, as was previously observed by Galeffi *et al.* (2000).

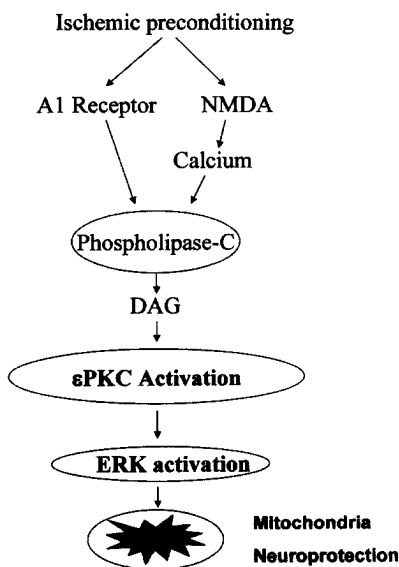


Fig. 2. Flow chart defining the signal transduction pathways leading to ischemic tolerance. We and others have characterized that the adenosine A1 and the NMDA receptors are involved in the triggering phase of IPC. The ensuing pathways lead to ϵ PKC and ERK activation, which we propose may be protecting mitochondria.

Putative Role of the Signal Transduction Pathway on Mitochondrial Protection After Cerebral Ischemia: Role of Protein Kinase C (PKC)

We recently demonstrated that one specific PKC isozyme, namely ϵ PKC, plays a pivotal role in the induction of tolerance after ischemic preconditioning (Lange-Asschenfeldt *et al.*, 2003; Raval *et al.*, 2003; Raval and Perez-Pinzon, 2003) (see Fig. 2). Basu *et al.* (2002) showed that cleavage of ϵ PKC by caspase-7 results in the activation of ϵ PKC, which was associated with its antiapoptotic function. Also, formation of mitochondrial ϵ PKC-ERK $^{1/2}$ modules was coupled to the inactivation of BAD, a proapoptotic molecule (Baines *et al.*, 2002). Since, ischemic preconditioning has been shown to preserve mitochondrial function (Dave *et al.*, 2001; Fryer *et al.*, 2000), we hypothesize that ϵ PKC promotes ischemic tolerance by protecting mitochondrial function during the reperfusion phase. However, the precise mechanism by which IPC via ϵ PKC protects mitochondria following cerebral ischemia remains undefined. Further studies from our laboratory are underway to define these mechanisms.

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Mitochondrial Glutathione: A Modulator of Brain Cell Death

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The small fraction of glutathione in mitochondria in nonneural tissues is an important contributor to cell survival under some conditions. However, there has been only limited characterization of the properties and function of mitochondrial glutathione in cells from the brain. In astrocytes in culture, highly selective depletion of this glutathione pool does not affect cell viability, at least in the first 24 h, but does greatly increase susceptibility to exposure to nitric oxide or peroxynitrite. In vivo, a selective partial loss of glutathione develops during focal cerebral ischemia and persists during reperfusion. The timing and distribution of glutathione loss shows an apparent association with the likelihood that tissue infarction will subsequently develop. Furthermore, infarct volume is greatly decreased by intracerebroventricular infusion of glutathione monoethylester, a compound that can increase mitochondrial glutathione. Together these recent findings indicate that alterations in mitochondrial glutathione are likely to contribute to the severity of tissue damage in stroke and possibly other neurological disorders. Thus, this antioxidant pool provides a potentially useful target for therapeutic intervention.

KEY WORDS: Mitochondria; glutathione; astrocytes; focal cerebral ischemia; stroke; infarction; oxidative stress; cell death.

INTRODUCTION

Glutathione in cells is a major antioxidant acting both directly to remove reactive oxygen species and as a substrate for several peroxidases (Dringen, 2000). This tripeptide is also involved in other reactions including the conjugation of foreign molecules catalyzed by glutathione S-transferases. Cellular glutathione is found in two separate but interacting pools located in the cytoplasm and mitochondria. The cytoplasmic pool typically accounts for 85% or more of the total glutathione in cells (Griffith and Meister, 1985; Lash *et al.*, 1998; Meister, 1995). Most, if not all, of the synthesis of glutathione occurs in the cytoplasm (Griffith and Meister, 1985; Lash *et al.*, 1998; Martensson *et al.*, 1990). Thus, the long-term maintenance

of mitochondrial glutathione depends on transport from this site.

MITOCHONDRIAL GLUTATHIONE AND CELL VIABILITY

In many cells, glutathione in the mitochondria is much more important than the larger cytoplasmic pool in maintaining cell viability and limiting damage to various potentially toxic treatments. This key contribution of mitochondrial glutathione in preserving cell viability was first proposed by Meredith and Reed (1982, 1983) on the basis of the greatly increased death of hepatocytes when both glutathione pools were experimentally depleted compared to cells with losses of cytoplasmic glutathione alone. Subsequent studies identified similar responses in other types of cells and further showed that depletion of glutathione from the mitochondria was associated with much greater dysfunction and loss of viability in cells challenged with a range of oxidative stresses and other insults (Colell *et al.*, 1998; Colell *et al.*, 2001; Dhanboora and Babson, 1992; Fernandez-Checa *et al.*, 1991; Shan *et al.*, 1993; Wullner *et al.*, 1999). In many

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of the relevant investigations, the deleterious response to mitochondrial glutathione depletion was demonstrated on a background of cytoplasmic glutathione loss making it difficult to unequivocally evaluate the contribution of the mitochondrial pool alone. Selective partial depletion of mitochondrial glutathione has been achieved without accompanying changes in the cytoplasmic pool in only a few studies. Such cells also exhibited substantially decreased viability in response to substances including *t*-butylhydroperoxide (Fernandez-Checa *et al.*, 1991) and tumor necrosis factor (Colell *et al.*, 1998; Colell *et al.*, 2001) providing more direct evidence of the essential role of mitochondrial glutathione in preserving cell function.

These findings point to the potential for mitochondrial glutathione changes to contribute to tissue dysfunction and damage in disease states. Partial losses of mitochondrial glutathione develop in vivo under some conditions that include long-term ethanol feeding (Fernandez-Checa *et al.*, 1991) and liver ischemia with reperfusion (Grattagliano *et al.*, 1999). Glutathione loss induced in the liver by ethanol feeding is restricted to the mitochondrial pool. It results from a decreased glutathione uptake into mitochondria due to changes in the fluidity of the mitochondrial membranes (Coll *et al.*, 2003; Colell *et al.*, 1998; Colell *et al.*, 2001; Fernandez-Checa *et al.*, 1991). This partial loss of glutathione increases the susceptibility of the liver cells to several potentially toxic treatments (Colell *et al.*, 1998; Colell *et al.*, 2001; Fernandez-Checa *et al.*, 1991; Zhao *et al.*, 2002).

INTERACTIONS OF MITOCHONDRIAL AND CYTOPLASMIC GLUTATHIONE

The mechanisms of glutathione uptake into mitochondria and the control of this process have mostly been investigated in organelles isolated from the liver and kidney. The relevance of the findings to other tissues including the brain remains to be established. Glutathione carries a net negative charge. Its transport into mitochondria involves movement against the charge gradient across the inner mitochondrial membrane and therefore requires either energy or exchange with another anion. In kidney mitochondria, the 2-oxoglutarate and dicarboxylate transporters appear to be major contributors to glutathione uptake (Chen and Lash, 1998; Lash *et al.*, 2002). Transport into liver mitochondria shows some similarities but also significant differences in properties (Coll *et al.*, 2003; Martensson *et al.*, 1990; Meister, 1995), suggesting tissue specific characteristics. The 2-oxoglutarate transporter again seems to be involved (Coll *et al.*, 2003).

Although the mitochondria have an ongoing reliance on the cytoplasm for the supply of glutathione, these two pools can be separately modulated under some conditions. The glutathione synthesis inhibitor, buthionine sulfoximine, has been found in many different types of cells to substantially deplete cytoplasmic glutathione while having little initial impact on the mitochondrial pool (Martensson *et al.*, 1989; Meister, 1995). Indeed, treatment with this compound in vivo typically causes major losses of the cytoplasmic pool within a few hours in a range of tissues but only produces slow depletion of mitochondrial glutathione over many days or weeks. Partial selective losses of the mitochondrial glutathione pool also develop in response to some treatments of cells in vitro (Colell *et al.*, 1998; Colell *et al.*, 2001; Shan *et al.*, 1993) and in disease models in vivo (Anderson and Sims, 2002; Fernandez-Checa *et al.*, 1991; Wallin *et al.*, 2000). Thus, under the conditions examined in these studies, the transport processes do not replenish glutathione content in the mitochondria even though cytoplasmic glutathione is preserved.

MITOCHONDRIAL GLUTATHIONE IN CELLS FROM BRAIN

Despite the evidence for the important roles of mitochondrial glutathione in nonneural cells, surprisingly little attention has been given to the properties and functions of this antioxidant pool in cells from the central nervous system. Cerebellar granule neurons in culture exhibit marked functional deterioration and die in response to complete loss of both the mitochondrial and cytoplasmic glutathione but not with cytoplasmic glutathione loss alone (Wullner *et al.*, 1999). Thus, these neurons exhibit a similar dependence on mitochondrial glutathione to other cell types. Complete depletion of glutathione in motor neurons (Rizzardini *et al.*, 2003) and in astrocytes in culture (Huang and Philbert, 1996) also promotes cell dysfunction and death, although these studies did not relate changes in cell vulnerability to the responses of the individual glutathione pools.

We have recently established conditions based on ethacrynic acid treatment of cortical astrocytes in culture that result in complete loss of mitochondrial glutathione while leaving the cytoplasmic pool essentially unchanged. Ethacrynic acid has been widely used to produce total glutathione depletion in various cells, including neurons and astrocytes in culture. This compound is conjugated with glutathione in both the cytoplasm and mitochondria in reactions catalysed by some glutathione S-transferases. In neurons and astrocytes (Huang and Philbert, 1996;

Wullner *et al.*, 1999), but not in some nonneural cells (Meredith and Reed, 1982), ethacrynic acid produces more rapid depletion of the glutathione in the mitochondria than in the cytoplasm. This differential selectivity has not previously been exploited experimentally. Indeed, as far as we are aware, there are no previous reports for any cell type in which comparable selectivity of mitochondrial glutathione depletion has been achieved. Thus, this preparation provides a valuable tool for assessing the function of mitochondrial glutathione in a major population of cells derived from the brain.

Astrocytes with depleted mitochondrial glutathione showed no change in viability compared with nondepleted cells when incubated under normal conditions for 24 h. However, the glutathione-depleted astrocytes were much more susceptible to exposure to the peroxynitrite donor, 3-morpholininosydnonimine (Sin-1) and to nitric oxide (Muyderman *et al.*, 2004; Muyderman *et al.*, submitted). The cells treated with Sin-1 exhibited earlier and larger changes in cell function compared with astrocytes with preserved mitochondrial glutathione and also showed a substantial increase in cell death based on lactate dehydrogenase release and propidium iodide staining at both 3 and 24 h after treatment.

Interestingly, the glutathione content of the mitochondria recovered only partially over several hours following ethacrynic acid treatment despite the ongoing availability of glutathione in the cytoplasm. Thus, these cells showed much less rapid restoration of glutathione content than expected based on studies of isolated mitochondria from other tissues. This finding suggests either that the glutathione transport exhibits different properties in these cells or it has been modified as a result of the ethacrynic acid treatment. A slow recovery of partially depleted mitochondrial glutathione despite a preserved cytoplasmic pool has also been seen following treatment of COS cells with 4-hydroxynonenal (Raza *et al.*, 2002). This compound is also conjugated in a reaction catalyzed by glutathione S-transferase raising the possibility that the products of such reactions might interfere with glutathione uptake into the mitochondria.

Mitochondrial glutathione in the astrocytes treated with ethacrynic acid could be fully replenished using glutathione monoethylester as a precursor. Importantly, such restoration of the mitochondrial glutathione blocked the increased cell death resulting from exposure to Sin-1, clearly implicating the glutathione loss (and not some other consequence of ethacrynic acid treatment) as the basis for the greater vulnerability of these cells. The cell loss was also blocked by incubations with cyclosporin A, suggesting a role for induction of the mitochondrial permeability transition in the increased susceptibility of

glutathione-depleted astrocytes. The oxidation of specific protein sulfhydryls promotes induction of the permeability transition (Kowaltowski *et al.*, 2001), providing a likely link between glutathione depletion and this deleterious mitochondrial change.

TISSUE DAMAGE IN STROKE: A ROLE FOR MITOCHONDRIAL GLUTATHIONE DEPLETION?

In keeping with the relatively limited investigations of the role of mitochondrial glutathione in the central nervous system, there have been very few attempts to address whether mitochondrial glutathione is altered in neurological disorders. Transient selective depletion of glutathione in the mitochondria develops as an early change in a model of neonatal hypoxia-ischemia (Wallin *et al.*, 2000). We have also identified decreases in this antioxidant pool in a model of stroke in adult rats and provided evidence suggesting that this change could contribute to the tissue damage that develops in this major neurological disease. These changes and their possible implications for the pathology associated with stroke are briefly reviewed below.

Stroke in humans most commonly arises from blockage of an intracerebral artery. Although this occlusion is usually long-lasting or permanent, temporary occlusion is also seen in a subgroup of patients, particularly with recent increased use of thrombolytic agents to reverse the arterial blockage. The focal cerebral ischemia resulting from arterial occlusion typically produces immediate functional impairment. Unless the occlusion is reversed within the first hour or so, changes are initiated that lead over many hours to tissue infarction due to the death of all cell types within parts of the perfusion territory of the affected vessel. The location and volume of infarcted tissue are important determinants of the long-term symptoms of stroke. Thus, there has been considerable effort in recent years to elucidate the molecular events contributing to the ischemia-induced cell loss with a view to identifying therapeutic targets to limit tissue damage and improve outcome (Lipton, 1999; Sims and Anderson, 2002; Zheng *et al.*, 2003). These studies have provided evidence that interactions of multiple deleterious changes are required for cell death, with the exact mechanism being greatly influenced by factors including the severity of the ischemia and whether arterial occlusion is permanent or temporary.

Changes in mitochondrial function are one of the factors likely to contribute to ischemic cell death under at least some conditions (Sims and Anderson, 2002). The most direct mitochondrial effects arise from the reduced

delivery of oxygen to the tissue. The resultant impairment of the electron transport chain contributes to marked disruption of ATP and related metabolites in the most severely ischemic "core" or "focal" tissue within the perfusion territory of the affected vessel and also to lesser changes in surrounding perifocal tissue that is subjected to more moderate reductions in blood flow (Folbergrova *et al.*, 1992, 1995). Many of these metabolite changes substantially recover on reperfusion even in tissue destined to become infarcted but lactate often remains elevated, suggesting ongoing mitochondrial impairment. Cell susceptibility may be further compromised by direct decreases in mitochondrial capacity for respiratory function which develop during ischemia and reperfusion (Anderson and Sims, 1999; Kuroda *et al.*, 1996; Nakai *et al.*, 1997). Increased production of nitric oxide and its derivatives has been strongly implicated as a contributor to tissue damage in stroke (Eliasson *et al.*, 1999). One deleterious effect of these substances could result from their ability to inhibit the electron transport chain (Radi *et al.*, 2002). More direct support for a mitochondrial contribution to cell death is provided by the substantial protection achieved using a mitochondrial potassium channel opener in both permanent and temporary ischemia (Liu *et al.*, 2002; Shimizu *et al.*, 2002) and also by inhibitors of the mitochondrial permeability transition administered near the time of reperfusion in temporary ischemia (Matsumoto *et al.*, 1999; Yoshimoto and Siesjö, 1999).

We have identified a partial loss of glutathione in mitochondria isolated from ischemic brain regions in a rat model of stroke (Anderson and Sims, 2002). This change persisted for at least several hours of reperfusion. The glutathione losses during ischemia were not accompanied by changes in total tissue glutathione and were only seen with ischemic periods sufficient to induce subsequent infarction. Indeed, the time at which mitochondrial glutathione loss was first detected during ischemia in focal tissue from the striatum and cortex and in cortical perifocal tissue corresponded with the ischemic times typically required to initiate infarct formation in these same regions. This association is consistent with the mitochondrial glutathione loss being one factor contributing to the tissue damage, perhaps by providing conditions promoting induction of the permeability transition or other deleterious mitochondrial changes.

The glutathione depletion in ischemia was temporarily blocked or reversed by a single intracerebral injection of glutathione monoethylester but this treatment did not modify infarct volume (Anderson *et al.*, 2004b). However, more prolonged treatment with this glutathione ester via intracerebroventricular infusion initiated at the time of temporary arterial occlusion reduced infarct volume by

more than 60% (Anderson *et al.*, 2004a). Further studies are needed to demonstrate that mitochondrial glutathione is indeed contributing to this protective response and to identify the critical period(s) during which the glutathione ester is protective. Nonetheless, the results obtained so far are consistent with mitochondrial glutathione loss being an important factor in the vulnerability of cells in ischemic and postischemic brain.

IMPLICATIONS FOR NEUROPROTECTION

The findings from investigations of the brain in vivo and of cell populations derived from this tissue are consistent with a key role for mitochondrial glutathione in promoting cell viability under some pathological conditions. Additional studies are needed to better understand the normal controls influencing the content of glutathione in brain mitochondria and to further evaluate the possible contribution of mitochondrial glutathione changes in stroke and other brain disorders. Findings to date indicate that treatments to replenish or increase this endogenous antioxidant pool might limit cell death in some brain disorders and could possibly be useful as a prophylactic treatment in situations (such as major surgery) that are associated with an increased risk of brain damage.

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Mitochondrial Enzymes and Endoplasmic Reticulum Calcium Stores as Targets of Oxidative Stress in Neurodegenerative Diseases

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Considerable evidence indicates that oxidative stress accompanies age-related neurodegenerative diseases. Specific mechanisms by which oxidative stress leads to neurodegeneration are unknown. Two targets of oxidative stress that are known to change in neurodegenerative diseases are the mitochondrial enzyme α -ketoglutarate dehydrogenase complex (KGDHC) and endoplasmic reticulum calcium stores. KGDHC activities are diminished in all common neurodegenerative diseases and the changes are particularly well documented in Alzheimer's disease (AD). A second change that occurs in cells from AD patients is an exaggerated endoplasmic reticulum calcium store [i.e., bombesin-releasable calcium stores (BRCS)]. H_2O_2 , a general oxidant, changes both variables in the same direction as occurs in disease. Other oxidants selectively alter these variables. Various antioxidants were used to help define the critical oxidant species that modifies these responses. All of the antioxidants diminish the oxidant-induced carboxy-dichlorofluorescein (cDCF) detectable reactive oxygen species (ROS), but have diverse actions on these cellular processes. For example, α -keto- β -methyl-*n*-valeric acid (KMV) diminishes the H_2O_2 effects on BRCS, while trolox and DMSO exaggerate the response. Acute trolox treatment does not alter H_2O_2 -induced changes in KGDHC, whereas chronic treatment with trolox increases KGDHC almost threefold. The results suggest that KGDHC and BRCS provide targets by which oxidative stress may induce neurodegeneration and a useful tool for selecting antioxidants for reversing age-related neurodegeneration.

KEY WORDS: Reactive oxygen species; KGDHC; bombesin-releasable calcium stores; Alzheimer's disease; oxidants; antioxidants.

INTRODUCTION

Overwhelming evidence indicates that damage from reactive oxygen species (ROS) occurs in Alzheimer's disease (AD) brain (Markesbery *et al.*, 1999; Smith *et al.*, 2000). For example, the reactive aldehyde acrolein is present throughout the AD brain. Many or most tangles contain acrolein; however, acrolein is also present in brain areas that do not have tangles, suggesting that oxidative stress is more pervasive than tangles (Calingasan *et al.*, 1999). The presence of hydroxynonenal, another reactive aldehyde (Markesbery *et al.*, 1999), also indicates

that reactive aldehydes and lipid damage is extensive in the brain. Protein oxidation (Aksenov *et al.*, 2001; Lyras *et al.*, 1997) and nitration (Smith *et al.*, 1997) indicate damage to protein. ROS-induced damage to all four DNA bases and to RNA (8-hydroxyguanosine) occurs in brains from AD patients (Lyras *et al.*, 1997). The presence of ferritin, hemoxygenase, and reactive iron are indicators of ongoing oxidative stress (Rottkamp *et al.*, 2001). A recent study of autopsy brains suggests that measures of oxidative stress are greatest early in the disease and then decline (Nunomura *et al.*, 2001). Although the inability to make temporal measurements in autopsy brain makes mechanistic approaches equivocal, results in transgenic mice also indicate that oxidative stress is an early change. In these mice, β -amyloid (A β) is deposited in brain because of overexpression of a human amyloid

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precursor protein transgene with a double mutation found in a Swedish family with early-onset AD. Measures of oxidative stress (isoprostane levels) in these mice precede the surge of A β production and plaque formation by more than a month (Pratico *et al.*, 2001). Whether a similar, early increase in isoprostanes occurs in human brain is unknown. The specific targets of this oxidative stress and how the changes might be reversed is the focus of this review.

THE α -KETOGLUTARATE DEHYDROGENASE ENZYME COMPLEX (KGDHC) AND NEURODEGENERATIVE DISEASE

Brain metabolism is diminished in AD, and a possible underlying cause of the decline is a reduction in the activity of the KGDHC. KGDHC consists of three proteins: E1k, E2k, and E3. KGDHC is a key and arguably rate-limiting enzyme of the tricarboxylic acid cycle. KGDHC is thiamine-dependent, and is diminished in the brains of individuals with thiamine deficiency (i.e., Wernicke-Korsakoff patients), who have severe memory deficits (Butterworth *et al.*, 1993). Several groups report that KGDHC is diminished in brains from patients with AD, and no contravening reports exist (Gibson *et al.*, 2000). The AD-related reduction in KGDHC activity occurs in genetic and nongenetic forms of AD. Diminished activities occur in brain regions with severe pathology, as well as in areas that show minimal pathology. In the non-genetic forms of the AD, the immunoreactivity of these three components is not altered (Mastrogiacomo *et al.*, 1993), whereas in genetic forms of AD, protein levels of E1k and E2k, but not E3 decline (Gibson *et al.*, 1997). This suggests that the enzyme can be inactivated by multiple mechanisms (Fig. 1). The relation of the decline in KGDHC to the pathophysiology of AD varies with apolipoprotein geno-type. In patients with one apolipoprotein E4 allele, the correlation of KGDHC activities to the clinical dementia rating is very high ($r = 0.7$). In this same subpopulation, the correlation with plaques ($r = 0.11$) and tangles ($r = 0.32$) is very low (Gibson *et al.*, 2000). The decline in KGDHC also occurs in brain regions away from the regions of pathology, indicating that the reduction is not just secondary to neurodegeneration. Further, the decline does not appear to be a general loss in mitochondrial function because another mitochondrial enzyme, glutamate dehydrogenase, is unchanged. Thus, brain KGDHC activities decline with AD, and the changes appear to be pathophysiologically important.

KGDHC is diminished in several neurodegenerative disorders. These include progressive supranuclear palsy

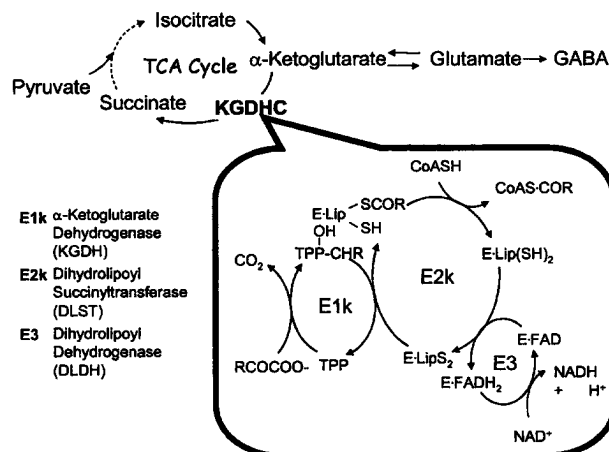


Fig. 1. KGDHC and metabolism.

(Park *et al.*, 2001), Parkinson's disease (Gibson *et al.*, 2003), and Huntington's disease (Klivenyi *et al.*, 2004). As in AD, the decline occurs in both areas of neurodegeneration and in other brain areas. Further, the results are consistent with KGDHC being more sensitive to the disease process (e.g., some undetectable form of ROS than other common measures of oxidative stress). For example, a decline in cerebellar KGDHC in PSP patients occurs although there is not an increase in malondialdehyde, protein carbonyl formation, or protein nitration (Park *et al.*, 2001).

CONSEQUENCES OF DIMINISHED KGDHC ACTIVITIES

Diminishing KGDHC activities has profound consequences on cell and brain function. In cells, inhibition of KGDHC correlates with release of cytochrome C and activation of caspase pathways, and these events precede alterations in the mitochondrial membrane potential (Huang *et al.*, 2003). In brain slices, inhibition of acetylcholine synthesis is particularly sensitive to KGDHC inhibition (Gibson and Blass, 1976). Two experimental approaches suggest that diminished KGDHC activities do not induce the neurodegeneration, but predispose to damage by other means. The first approach is thiamine deficiency, which leads to selective neurodegeneration. A thiamine derivative is a cofactor for KGDHC, and a major consequence of thiamine deficiency is a decline in the activity of KGDHC. However, neither the distribution of KGDHC nor the response of KGDHC to neurodegeneration suggests that KGDHC is directly responsible for neuron death. Instead, the results suggest that the decline in KGDHC predisposes to other insults that promote the neuronal death

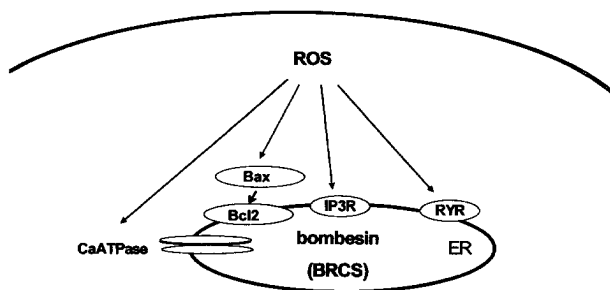


Fig. 2. Bombesin-releasable calcium stores (BRCS).

(Sheu *et al.*, 1998). A second approach to test the consequences of a diminished KGDHC activity is to use transgenic mice that have reduced levels of the E3 component of KGDHC, and thus diminished KGDHC activities. These mice did not show any pathological changes. However, lesions induced by MPTP and 3-NP were much larger in these mice (Klivenyi *et al.*, 2004).

EXAGGERATED ENDOPLASMIC RETICULUM STORES OF CALCIUM OCCUR IN AD AND IN ANIMAL MODELS OF AD

A second change that accompanies AD is an exaggeration in the internal pools of calcium. Both mitochondrial (Gibson *et al.*, 1997) and endoplasmic reticulum (Gibson *et al.*, 1996; Ito *et al.*, 1994) stores differ between cells from AD patients and controls. The increase in $[\text{Ca}^{2+}]_i$ after the addition of bombesin or bradykinin in the absence of calcium is used to assess endoplasmic reticulum stores of calcium. Experimentally, these are defined as bombesin or bradykinin releasable calcium stores (BRCS). The BRCS are exaggerated in fibroblasts from AD patients (Ito *et al.*, 1994). The increases in these pools

appear related to the changes in capacitative calcium entry (Leissring *et al.*, 2000; Yoo *et al.*, 2000) and to the reduced flux of calcium into the cells (Peterson *et al.*, 1985). BRCS has been examined in fibroblasts from multiple individuals with AD, including non-genetic forms of AD, and in those bearing PS1 (Ito *et al.*, 1994) and APP (Gibson *et al.*, 1997) mutations. Increases in BRCS also occur in cells that have been transfected with mutant PS-1 (Guo *et al.*, 1996) and in both fibroblasts and neurons from transgenic mice bearing a presenilin-1 mutation (Leissring *et al.*, 2000). Thus, many approaches indicate that this calcium store is altered in cells from AD patients. The possible actions of ROS on the BRCS are shown in Fig. 2.

KGDHC AS A TARGET OF OXIDATIVE STRESS AND FOR REVERSAL WITH ANTIOXIDANTS

Oxidants produce inactivation of KGDHC just as seen in AD. KGDHC is inactivated by a variety of oxidants including peroxynitrite, NO (Park *et al.*, 1999), hydroxyl-nonenal (Humphries *et al.*, 1998), H_2O_2 (in mM concentrations), chloroamine (μM concentrations), and sodium hypochlorite (in nM concentrations). H_2O_2 diminishes KGDHC activity in synaptosomes (Chinopoulos *et al.*, 1999), fibroblasts (Gibson *et al.*, 2002), and N2a cells. Although all forms of AD have reduced brain KGDHC activities, protein levels as determined by immunoreactivity decline in some forms of AD but not others. Even related oxidants can produce a dichotomy similar to that which occurs in AD brain. Both NO and peroxynitrite diminish KGDHC activities. However, peroxynitrite, but not NO, diminishes immunoreactivity of E1k and E2k. The first pattern is similar to that observed in AD patients bearing the APP670/671 mutation (Table I).

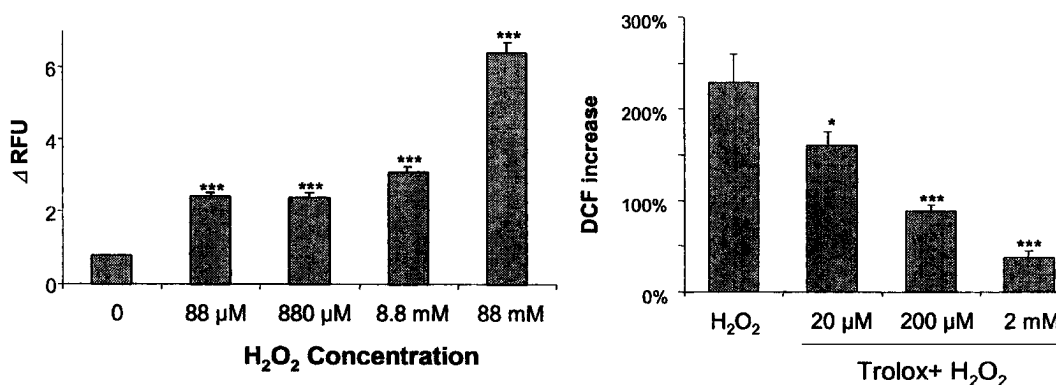


Fig. 3. H_2O_2 -induced increase in ROS is sensitive to Trolox (1-h. treatment). Asterisks indicate difference from the control groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table I. Selective Changes in KGDHC Immunoreactivity with Oxidative Stress

	E1k	E2k	E3
NO(SNP)	↔	↔	↔
Peroxynitrite	↓	↓	↔

The second pattern is similar to that seen in AD patients with no known genetic basis (Mastrogiacomo *et al.*, 1993).

KGDHC activity is reduced in animal and cell models with increased oxidative stress. Transgenic superoxide dismutase (SOD2) knockout mice have reduced KGDHC activities in their brains (Hinerfeld *et al.*, 2004). KGDHC is diminished. In cells that overexpress monoamine oxidase (MAO), and increased substrate (i.e., more ROS) exaggerates the reduction in KGDHC (Kumar *et al.*, 2003).

The sensitivity of KGDHC to ROS suggests that antioxidants should protect KGDHC. The interactions with KGDHC have been studied with H₂O₂. H₂O₂ produces a dose-dependent increase in reactive oxygen species as detected with cDCF. Trolox reduces the ROS production in a dose-dependent manner (Gibson *et al.*, 2002) (Fig. 3). However, under these same conditions, trolox does not protect KGDHC against H₂O₂ in acute condition (Fig. 4). This was also true of the antioxidants *N*-acetylcysteine and DMSO. On the other hand, if cells are incubated with trolox for 5 days, there was a dose-dependent increase in KGDHC. Following H₂O₂ treatment, all trolox-treated cells had higher KGDHC activities than control cells or H₂O₂ alone (Fig. 4). The intermediate concentration of trolox diminishes the H₂O₂-induced reduction in KGDHC activities by 50% (Gibson *et al.*, 2002). These results suggest that under some conditions (e.g. acute or chronic trolox treatment with some antioxidants), it is possible to protect KGDHC.

Table II. Select Changes in BRCS with Various Oxidants

	DCF-ROS	Change in BRCS
H ₂ O ₂	++	+
SIN-1	+++++	0
<i>t</i> -BHP	+++	++
HX/XO	+++++	-
SNAP	+++	++
SNP	++	0

BRCS AS A TARGET OF OXIDATIVE STRESS AND REVERSAL WITH ANTIOXIDANTS

BRCS are selectively altered by various oxidants (Table II). BRCS is increased by *tert*-butyl-hydroperoxide (*t*-BHP), H₂O₂, and *S*-nitroso-*N*-acetylpenicillamine (SNAP), is unaffected by 3-morpholinomethyl-*N*-nitrosodimethylamine (SIN-1) or sodium nitroprusside (SNP), and is diminished by hypoxanthine/xanthine oxidase (HX/XO). These oxidants produce ROS that can be distinguished with other fluorescent indicators, including DAF and amplex red (data not shown).

The interactions of the antioxidant trolox with H₂O₂-induced changes in BRCS are not predictable (Fig. 5; Gibson *et al.*, 2002). Under the conditions that reduce H₂O₂-induced cDCF-detectable ROS, trolox exaggerates H₂O₂-induced (88 μ M) increases in BRCS. However, chronic treatment with trolox has no effect on BRCS (i.e., nearly the opposite effects that are observed with KGDHC). Acute treatment with di-methylsulfoxide (DMSO) also exaggerates the BRCS, while NAC has no effect (data not shown). An understanding of the interaction of various oxidant species with various antioxidants will possibly reveal which species lead to the AD-like changes in BRCS.

Further experiments tested the ability of α -keto- β -methyl-*n*-valeric acid (KMV) (20 mM) to reduce

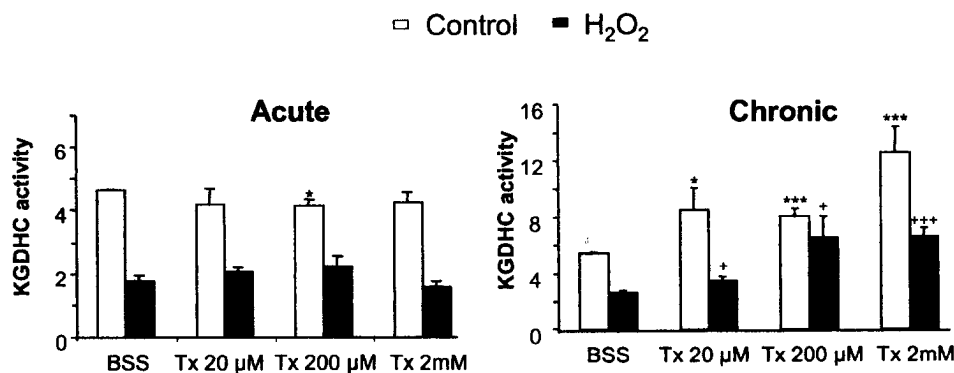


Fig. 4. Trolox provides limited protection of KGDHC from H₂O₂. Asterisks indicate difference from the control groups: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

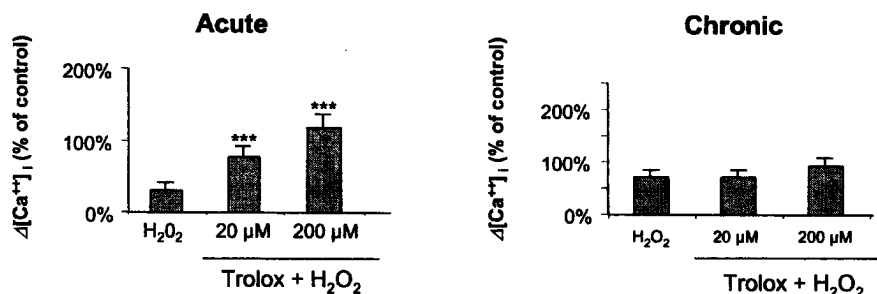


Fig. 5. Trolox exaggerates the effects of H_2O_2 on BRCS. H_2O_2 (final $88 \mu\text{M}$) was added in both acute and chronic trolox treatments. Asterisks indicate difference from the control groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

c-DCF-detectable ROS and interact with BRCS (Fig. 6). KMV appears to be a unique antioxidant. KMV diminishes c-DCF-detectable ROS that are induced by H_2O_2 , hypoxia, and SIN-1, but does not neutralize 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF)-detectable NO induced by SIN-1. KMV reduces both BRCS and the H_2O_2 -induced change in BRCS. Thus, KMV is better than trolox at protecting against H_2O_2 when BRCS is regarded as the target. The results suggest that the same H_2O_2 -induced ROS that reacts with KMV may also underlie the changes in BRCS related to AD.

CONCLUSIONS

Oxidative stress is a common feature of neurodegenerative diseases. This may lead to oxidation of key components of BRCS and of KGDHC. The disease-related changes in these processes in disease can be patterned by select oxidants. The results suggest these changes may be important in normal signaling of the molecules as well in neurodegeneration. Therefore, reversal of the changes in these processes by select antioxidants may be beneficial.

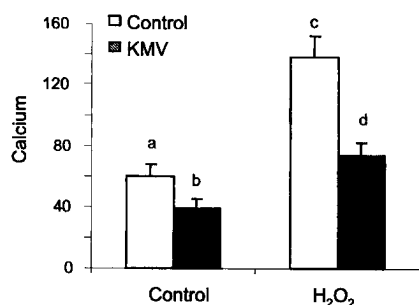


Fig. 6. KMV protects BRCS against H_2O_2 . H_2O_2 ($88 \mu\text{M}$) was added after 1 min basal $[\text{Ca}^{2+}]_i$ measurements in the presence of KMV (20 mM). Data are means \pm SEM. Values with different letters denote significance ($P < 0.05$).

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Mitochondrial Nitric-Oxide Synthase: Enzyme Expression, Characterization, and Regulation

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Nitric oxide is generated in vivo by nitric-oxide synthase (NOS) during the conversion of L-Arg to citrulline. Using a variety of biological systems and approaches emerging evidence has been accumulated for the occurrence of a mitochondrial NOS (mtNOS), identified as the alpha isoform of neuronal or NOS-1. Under physiological conditions, the production of nitric oxide by mitochondria has an important implication for the maintenance of the cellular metabolism, i.e. modulates the oxygen consumption of the organelles through the competitive (with oxygen) and reversible inhibition of cytochrome c oxidase. The transient inhibition suits the continuously changing energy and oxygen requirements of the tissue; it is a short-term regulation with profound pathophysiological consequences. This review describes the identification of mtNOS and the role of posttranslational modifications on mtNOS' activity and regulation.

KEY WORDS: Nitric oxide; nitric-oxide synthase; mitochondria; posttranslational modifications; oxygen consumption; oxygen.

INTRODUCTION

Nitric oxide is formed from L-arginine by nitric-oxide synthase (NOS), which oxidizes the guanidino nitrogen of arginine, releasing nitric oxide and citrulline (Bredt and Snyder, 1990, 1994). Three main NOSs are expressed in mammals and differ in their functions, amino acid sequence, posttranslational modification, and cellular location. Two NOS, neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3), are constitutively expressed and involved in signal cascades (Bredt and Snyder, 1990; Mayer *et al.*, 1989, 1990). The third NOS is cytokine-inducible (iNOS or NOS-2) and functions as both a regulator and effector of the immune response (Förstermann *et al.*, 1992; Stuehr and Marletta, 1987). The consequence to this diversity of location and function is a specific regulation of each isoform. For example, NOSs differ significantly regarding Ca^{2+} levels required to bind calmodulin, which triggers heme reduc-

tion and nitric-oxide synthesis (Abu-Soud *et al.*, 1994; Panda *et al.*, 2001). They also have different capacities to be up- or downregulated by Ser/Thr phosphorylation (Fulton *et al.*, 1999; Harris *et al.*, 2001). Using rat liver, we provided unequivocal evidence for a localization of a NOS (mitochondrial NOS or mtNOS) at the inner membrane of mitochondria (Giulivi *et al.*, 1998; Tatoyan and Giulivi, 1998). Given NOS' diverse biochemical characteristics, it could be postulated that the production of nitric oxide by mitochondria is highly regulated because of the critical role that this molecule has on cellular respiration (Brown and Cooper, 1994; Cleeter *et al.*, 1994; Poderoso *et al.*, 1996). In the following sections, the identification of mtNOS and the role of posttranslational modifications on its activity and regulation are summarized and discussed.

PRODUCTION OF NITRIC OXIDE BY MITOCHONDRIA

Our studies were the first providing evidence for production of nitric oxide by *purified* mitochondria (Giulivi, 1998; Giulivi *et al.*, 1998, 1999; Tatoyan and Giulivi, 1998; French *et al.*, 2001). This production of nitric oxide was demonstrated by using direct (L-citrulline production, evaluated by using colorimetric or radiolabeled

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compounds) and indirect techniques (nitric-oxide generation, evaluated by electron paramagnetic resonance with spin trapping; nitric oxide-dependent oxidation of oxymyoglobin). More evidence was furnished by the measurement of NOS activity in mitochondria isolated from purified hepatocytes, (abrogating the putative contamination of Kupffer cells), mitoplasts (mitochondria stripped of the outer membrane). Isolation and purification of mtNOS, mainly localized at mitochondrial membranes, allowed obtaining critical kinetic constants and dependence on cofactors and cosubstrates (Giulivi, 2003; Tatoyan and Giulivi, 1998).

Other labs had reached similar conclusions or extended the knowledge in this field in terms of the occurrence of a mtNOS by using less purified mitochondrial fractions, isolated cells, and various approaches such as colocalization of mitochondria with a production of nitric oxide in intact cells, nitric-oxide detection by electrode in wild-type and nNOS-KO mice mitochondria, among others (Bates *et al.*, 1995, 1996; Dedkova *et al.*, 2004; Ghafourifar and Richter, 1997; Kanai *et al.*, 2001; Kobzik *et al.*, 1995; López-Figueroa *et al.*, 2000).

Considering that the constitutive forms of nitric-oxide synthase, i.e., nNOS and eNOS, account for the rapid, transient, calcium-dependent production of nitric oxide (Bredt and Snyder, 1990; Mayer *et al.*, 1989, 1990), thus, it would be expected that increases in mitochondrial calcium be required to activate mtNOS. In line with this assumption, stimulation of nitric-oxide production by mtNOS was observed by bolus additions of calcium to mitochondria (Ghafourifar and Richter, 1997). The authors proposed that uptake of calcium by respiring mitochondria may lead to increased peroxynitrite formation in mitochondria, which in turn causes calcium release (Schweizer and Richter, 1996) via the pyridine nucleotide-dependent pathway (Lötscher *et al.*, 1979) followed by mtNOS deactivation. These observations have been interpreted as part of a feedback loop, which prevents calcium overloading and allows its release preserving membrane potential (Ghafourifar and Richter, 1997).

An apparent discrepancy arises in terms of the role of calcium on the rate of oxygen consumption by mitochondria: on one hand, mitochondrial calcium increases the rate of oxygen consumption as a result of the activation of calcium-activated dehydrogenases (Denton and McCormack, 1985, 1990, 1993; Hansford, 1985; McCormack *et al.*, 1990), and on the other, by activating mitochondrial nitric-oxide synthase, decreases the oxygen consumption by inhibiting cytochrome oxidase activity. When we evaluated the rates of State 3 oxygen consumption in the presence of N^G -monomethyl-L-Arg (NMMA), a competitive inhibitor of mtNOS, at various concentra-

tions of calcium, the $K_{0.5}$ was $0.1 \mu\text{M}$ (similar to that required for the activation of the Krebs' cycle) whereas in those with L-Arg (in which mtNOS was saturated with L-Arg) the $K_{0.5}$ was $0.45 \mu\text{M}$ (Traaseth *et al.*, 2004). By plotting the difference between the rates of oxygen consumption in State 3 with L-Arg and with NMMA at various calcium concentrations, a $K_{0.5}$ of $0.3 \mu\text{M}$ was obtained, similar to the $K_{0.5}$ ($0.26 \mu\text{M}$) of the dependence of the rate of nitric-oxide production on calcium concentrations, and within the values of other $K_{0.5}$ found for purified nNOS (Bredt and Snyder, 1992; Mayer *et al.*, 1989). Thus, the difference between these $K_{0.5}$ indicates that the activation of dehydrogenases, followed by the activation of mtNOS would result in the modulation of the Krebs' cycle activity by the modulation of nitric oxide on the respiratory rates (Traaseth *et al.*, 2004). This would ensue in changes in the NADH/NAD⁺ and ATP/ADP ratios, which would influence the rate of the cycle and the oxygen diffusion.

BIOCHEMISTRY OF MITOCHONDRIAL NITRIC-OXIDE SYNTHASE

The identification of mtNOS was a critical step in this research because it allowed the assignment of this protein to one of the known isoforms, or to depict it as a novel isoform. By using nitric-oxide electrodes to follow the production of nitric oxide by mitochondria, mtNOS has been identified as the nNOS, for nNOS-KO mice have no mtNOS (Kanai *et al.*, 2001). Independently, our lab reached the same conclusions in terms of identifying mtNOS as the alpha isoform of nNOS and expanded this concept to identify the isoform and posttranslational modifications (Elfering *et al.*, 2002). Briefly, purified mtNOS was separated by 2D-electrophoresis, followed by in-gel digestion with either trypsin or endoproteinase V8, and MALDI-ToF analyses were performed on the eluted fragments. The resulting sequences were blasted against in-silico trypsin- or V8-digested proteins from the PDB and matched to sequences of constitutive rat nNOS (Elfering *et al.*, 2002). Given that mouse bNOS has five isoforms (known as bNOS-1, bNOS2, bNOS-beta, bNOS-gamma, and bNOS-MU or muscle-specific; Brenman *et al.*, 1997; Ogura *et al.*, 1993; Silvagno *et al.*, 1996) produced by alternative splicing of mRNA, the question remained whether mtNOS was one of these products or represented a novel alternative splicing product. Some of the fragments obtained with MALDI excluded NOS-gamma and NOS-2, NOS-beta and NOS-gamma seemed unlikely candidates based on their MW, suggesting that either NOS-1 or -MU was mtNOS. RT-PCR experiments performed on enriched poly(A)⁺ mRNA from rat liver (using primers based on

MALDI-ToF sequences or gene-specific) and PCR experiments performed on rat liver cDNA resulted in the amplification of segments of the transcript corresponding to nNOS alpha isoform.

Our results combined from MALDI, MW, and pI indicated that mtNOS is bNOS, excluding the possibility of a novel isoform or an alternative splicing product. The identification of this enzyme was crucial because it will allow studying its biochemistry in detail with the previous knowledge that we have on the bNOS isoform.

Other studies had emerged perceiving mtNOS as a novel or other than the nNOS isoform as indicated above (Brookes, 2004, and references therein). Distinction needs to be made on what is considered identification parameters for a protein in biochemistry. Protein characterization (not identification) based on Western blotting technique is based on the crossreactivity of an antibody with a small segment of the protein (or epitope), which is usually smaller than the actual antigen used for immunization (usually 1 to 15% of the protein). Thus, a positive result in a Western blot should be understood as the crossreactivity of an antibody with a certain epitope in the protein, not necessarily indicating 100% homology with the rest of the protein. Considering that proteins like eNOS, iNOS, and nNOS have a 49 to 56% homology and that most of the commercially available antibodies are directed to the C-terminal half of the enzyme (or reductase domain) which shows pronounced sequence similarities to cytochrome P450 reductase (Bredt *et al.*, 1991) and where most of the homology among NOSs is present (48 to 55%) provides limited evidence for identification purposes. Even the combination of Western blotting with MW calculated from SDS-PAGE (where the error associated with proteins with a MW higher than 100 kDa is expected to be higher than 10% because of the limited availability of high MW standards and the lack of linearity between MW and mobility in this range, aside from the error constituted by the presence of posttranslational modifications such as acylation or glycosylation; Laemmli, 1970; Weber and Osborn, 1969) furnish inadequate information for identification purposes.

POSTTRANSLATIONAL MODIFICATIONS OF mtNOS AND THEIR ROLE IN NITRIC-OXIDE REGULATION

Acylation Pattern of Mitochondrial Nitric-Oxide Synthase

All three NOSs (i.e., n-, e-, and mac-NOSs) were recovered from primary cells in both a soluble and a partic-

ulate fraction (Hecker *et al.*, 1994; Liu and Sessa, 1994; Pollock *et al.*, 1991). Endothelial cNOS, which was recovered from resting cells almost exclusively in the latter fraction (Pollock *et al.*, 1991), was labeled when host cells were incubated with radioactive myristic acid (Liu and Sessa, 1994). Here, the amino-terminal group was co-translationally linked to myristic acid, supported by the following evidences: (1) the inhibition of myristic acid incorporation by a mutation of the amino-terminal Gly indicating that the fatty acid was bound by an amide bond to the protein; (2) cycloheximide treatment abolished the incorporation of myristic acid indicating that the fatty acid was incorporated during protein synthesis; and (3) the identification of myristic acid was performed by the release of myristoyl methyl ester from the protein, after acid methanolysis followed by hydroxylamine treatment. Later, it was reported that eNOS is also palmitoylated at the Cys residues. These acylations allowed the detection of the enzyme close to calveolin, located at the plasma membrane, in intact endothelial cells (Busconi and Michel, 1993). With bNOS isolated from rat but not rabbit, most of the protein sedimented in a subcellular fraction whose marker enzymes were typical of endoplasmic reticulum (Liu and Sessa, 1994). mtNOS was found mainly localized at the inner mitochondrial membrane, requiring the presence of CHAPS to solubilize the enzyme from mitochondrial membranes, indicating that the enzyme is tightly bound to the membrane. Experiments designed to investigate the putative acylation of mtNOS resulted in the finding that myristic acid is linked to mtNOS through an oxy- or thio-ester bond. Myristic acid was probably bound during a reversible, posttranslational process, catalyzed by acyltransferases. It should be noted that the pattern of acylation found with mtNOS differs from that observed with eNOS, in which an *N*-terminal myristoylation and palmitoylation of Cys residues were found. No data on acylation are available for the other two NOS, i.e., bNOS and macNOS; however, their main soluble localization may indicate a low or negligible acylation.

The occurrence of lipid-protein linkages in mtNOS may indicate an alternative modulatory role based on acylation-deacylation processes. The exact function of acylating proteins is still not known, and in this context, protein-protein interactions, membrane localization, or subcellular distribution has been proposed. In this case, it could be speculated that acylation of mtNOS is implicated in the regulation of mitochondrial nitric-oxide production. If acylated mtNOS could be incorporated to the mitochondrial membranes, this localization might be advantageous for the following reasons: first, nitric oxide will be produced closer to the target site, cytochrome oxidase, thus minimizing secondary reactions; second, it will

extend the lifetime of nitric oxide considering that this molecule when is produced in an aqueous, aerated solvent is consumed faster than if it is produced close to or in the membrane because nitrosyl dioxy—the product of nitric oxide and oxygen—can be stabilized by H bonds in an aqueous milieu (Beckman, 1996); and third, it may facilitate the targeting of the protein to mitochondria given that hydrophobic substrates tend to concentrate in these organelles.

Of note, proteins modified by ester bonds are potentially subject to dynamic regulation: the linkage and cleavage of palmitic acid to proteins are catalyzed by yet uncharacterized palmitoylthiotransferases and palmitoylthioesterase (Resh, 1994). These processes may underlie the ability of the enzyme, as with eNOS, to form stable, but dynamically regulated, associations with cell membranes (Busconi and Michel, 1993). This type of regulation could be also present in mitochondria and added a type of regulation to enzymatic activity by controlling the compartment in which the enzyme is present.

Phosphorylation of mtNOS

In response to agonists, eNOS accumulated phosphate and became soluble (Michel and Busconi, 1993) probably by decreasing the positive charge of a region that contributed electrostatically to the binding of eNOS to lipid. Other studies provided evidence that all three NOS isoforms immunoprecipitated from host cells are phosphorylated (Dawson *et al.*, 1993; Michel and Busconi, 1993). It has been shown that kinase- and phosphatase-dependent events occurring in cells modified NOSs activity (Bredt *et al.*, 1992; Brune and Lapetina, 1991; Nakane *et al.*, 1991). Recently, it has been reported that regulation of eNOS activity involves phosphorylation (Chen *et al.*, 1999; Dimmeler *et al.*, 1999) and coordinated signaling through Ser-1177 and Thr-495 by multiple protein kinases and phosphatases (Michell *et al.*, 2001).

Previous studies performed by our lab indicated that a phosphorylation was present in mtNOS at the fragment comprised between amino acids 1408 and 1421. Interestingly, Ser-1177 or 1179 from human or bovine eNOSs, respectively, which was found to play a critical role in the coordinated phosphorylation/dephosphorylation of the protein (Michell *et al.*, 2001), is homologous to Ser-1413 in rat bNOS, suggesting that this position could be subjected to phosphorylation. It is tempting to hypothesize that if ATP and respiratory substrate levels are high, then phosphorylation of mtNOS may enhance NOS' activity (by analogy with eNOS). This regulation will increase the production of nitric oxide, thus allowing an inhibition

of cytochrome oxidase and the consequent production of ATP. This pathway will allow that oxygen and other substrates get to cells that not necessarily are close to blood vessels, assuring a homogenous distribution.

PHYSIOLOGICAL ROLE OF mtNOS

We demonstrated that, under physiological conditions, the production of nitric oxide by mitochondria has an important implication for the maintenance of the cellular metabolism, i. e., nitric oxide (produced by rat liver mitochondria) modulated the oxygen consumption of the organelles (Giulivi, 1998, 2003; Giulivi *et al.*, 1999). This effect was achieved through the reversible inhibition of cytochrome oxidase by nitric oxide (Giulivi, 2003; Haynes *et al.*, 2003, and references therein). This transient inhibition suits the continuously changing energy and oxygen requirements of the tissue. However, if a sustained inhibition of cytochrome *c* oxidase is allowed, then other deleterious effects may happen: inhibition of ATP synthesis, release of cytochrome *c* (Ghafourifar *et al.*, 1999), increased oxygen radical production (Sarkela *et al.*, 2001), and nitration of critical biomolecules (Aulak *et al.*, 2001; Elfering *et al.*, 2004; Traaseth *et al.*, 2004).

Several lines of evidence indicated that the gaseous molecule nitric oxide by binding to the heme moiety of soluble guanylate cyclase leads to its activation, and the formation of cGMP triggers a variety of events in various organs (Dawson *et al.*, 1992; Garthwaite and Garthwaite, 1987; Marletta, 1989; Moncada *et al.*, 1991). However, our research and that of others had indicated cytochrome oxidase as a different target for nitric oxide, by which mediates other processes not mediated or triggered by cGMP. Our hypothesis is that nitric oxide produced by mitochondria has a short-term regulatory role on energy metabolism, oxygen consumption, and the inherent free radical production. The broader implications of the present work can help to redefine the way we view regulation of oxygen consumption in vivo. On the basis of our initial findings, it has been proposed that mitochondrial production of nitric oxide helps average oxygen utilization between cells at different distances from capillaries. The basic concept is that nitric oxide will slow oxygen consumption by cells closest to blood vessels, allowing oxygen to penetrate to cells at the boundary of becoming hypoxic. In addition, nitric oxide would help dilate blood vessels and potentially increase oxygen delivery to borderline hypoxic cells (Giulivi, 2003; Haynes *et al.*, 2003). Indeed, by following a similar line of thought, a mechanism for firefly flashing has been proposed in which the role of nitric oxide is to transiently inhibit mitochondrial

respiration in photocytes and thereby increasing the availability and level of oxygen in the peroxisomes, oxygen being the species considered as the biochemical trigger for light production (Trimmer *et al.*, 2001).

This emerging field in mtNOS is important, as it will expand the mechanisms by which cells consume oxygen and how changes in pO_2 are coped on a short-time framework. Studies in this field will provide key information on the molecular mechanisms of cellular respiration, and will likely lead to the design of better therapies to prevent pathological ischemic events during such diseases such as heart or brain stroke, and to advance our knowledge in the field of mitochondrial diseases.

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Protection Against Ischemic Brain Injury by Inhibition of Mitochondrial Oxidative Stress

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Mitochondria are both targets and sources of oxidative stress. This dual relationship is particularly evident in experimental paradigms modeling ischemic brain injury. One mitochondrial metabolic enzyme that is particularly sensitive to oxidative inactivation is pyruvate dehydrogenase. This reaction is extremely important in the adult CNS that relies very heavily on carbohydrate metabolism, as it represents the sole bridge between anaerobic and aerobic metabolism. Oxidative injury to this enzyme and to other metabolic enzymes proximal to the electron transport chain may be responsible for the oxidized shift in cellular redox state that is observed during approximately the first hour of cerebral reperfusion. In addition to impairing cerebral energy metabolism, oxidative stress is a potent activator of apoptosis. The mechanisms responsible for this activation are poorly understood but likely involve the expression of p53 and possibly direct effects of reactive oxygen species on mitochondrial membrane proteins and lipids. Mitochondria also normally generate reactive oxygen species and contribute significantly to the elevated net production of these destructive agents during reperfusion. Approaches to inhibiting pathologic mitochondrial generation of reactive oxygen species include mild uncoupling, pharmacologic inhibition of the membrane permeability transition, and simply lowering the concentration of inspired oxygen. Antideath mitochondrial proteins of the Bcl-2 family also confer cellular resistance to oxidative stress, paradoxically through stimulation of mitochondrial free radical generation and secondary upregulation of antioxidant gene expression.

KEY WORDS: Superoxide; nitric oxide; peroxynitrite; pyruvate dehydrogenase; calcium; apoptosis.

MITOCHONDRIAL TARGETS OF OXIDATIVE STRESS

Several lines of evidence indicate that oxidative stress is a primary mediator of neurologic injury following cere-

bral ischemia. The extent of delayed neuronal death correlates well with prelethal markers of oxidative molecular alterations. Neuroprotection is observed following the use of antioxidants and inhibitors of free radical producing enzymes, e.g., nitric-oxide synthetase. In addition, neuroprotection is evident in genetic animal models where genes coding for enzymes that promote oxidative stress are knocked down or out, and where genes coding for antioxidant enzymes, e.g., superoxide dismutase (SOD) are overexpressed (see (Lewen *et al.*, 2000) for review).

Virtually every cellular and extracellular molecular component is potentially sensitive to damage caused by oxidative stress. Oxidative modification to DNA, RNA, proteins, lipids, and small metabolites occur during ischemia/reperfusion. Our research focuses on the mitochondrion and its components as both targets and mediators of oxidative reperfusion injury (Fiskum *et al.*, 1999; Murphy *et al.*, 1999). From both in vitro studies with

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neural cells (Myers *et al.*, 1995) and animal models of global cerebral ischemia (Liu *et al.*, 1998), we conclude that mitochondrial energy metabolism is extremely sensitive to impairment by reactive oxygen and nitrogen species (ROS/RNS) and that mitochondrial oxidative stress limits metabolic recovery and promotes the intrinsic pathway of apoptosis.

One hypothesis we are testing is that during reperfusion, pyruvate dehydrogenase (PDH) is oxidatively modified and inactivated (Bogaert *et al.*, 2000; Rosenthal *et al.*, 1992), resulting in impaired oxidative energy metabolism and exacerbation of postischemic brain lactic acidosis (Rosenthal *et al.*, 1992). PDH enzyme activity is lost when purified enzyme is exposed to systems that generate hydroxyl radicals (Bogaert *et al.*, 1994) or peroxynitrite (E. Martin, unpublished), two ROS/RNS species strongly implicated in reperfusion brain injury. In addition to inactivation of PDH, the activity of the electron transport chain Complex I (NADH-CoQ oxidoreductase) is also depressed during reperfusion (Almeida *et al.*, 1995), which could be particularly important since this complex is normally the rate-limiting step of the electron transport chain (Davey *et al.*, 1997). The relative importance of damage to components of the electron transport chain compared to upstream metabolic enzymes, e.g., PDH, is at this juncture unknown. However, decreased production of NADH by PDH and TCA cycle dehydrogenases may be responsible for the hyperoxidized redox state of NAD(H) and components of the mitochondrial electron transport chain that occurs during the first hour of reperfusion after global cerebral ischemia (Rosenthal *et al.*, 1995). If the electron transport chain was metabolically rate limiting during reperfusion, the NAD(H) redox state should be relatively reduced rather than oxidized. Postischemic oxidation of NAD(H) may therefore constitute an important clue for the identification of the most important metabolic targets of reperfusion injury.

Other possible explanations for the effect of ischemia/reperfusion on NAD(H) redox state include depletion of pyridine nucleotides through PARP activation (Wang *et al.*, 2002), and release of mitochondrial NAD(H) and NADP(H) from the mitochondrial matrix into the cytosol, e.g., what occurs following activation of the mitochondrial membrane permeability transition (MPT) (Chinopoulos *et al.*, 2003). In addition to inhibiting PDH and other mitochondrial enzyme activities, ROS/RNS are potent activators of both PARP and the MPT (Kowaltowski *et al.*, 2000; Prabhakaran *et al.*, 2004). Moreover, the metabolism of H₂O₂ and other peroxides via the glutathione peroxidase/reductase system can contribute to the oxidative shift in pyridine nucleotide redox state. Irrespective of the mechanism by which cerebral reperfusion

causes this shift in redox state, the associated decrease in reducing power could limit detoxification of peroxides and maintenance of reduced protein sulfhydryl groups, thereby contributing to the prolonged oxidative stress characteristic of reperfusion tissue injury.

While oxidative damage to cerebral energy metabolism is a critical contributor to delayed, necrotic neuronal death, oxidative stress is also a powerful initiator of apoptosis, which also contributes significantly to ischemic neural cell death (DeGracia *et al.*, 2002; Hou and MacManus, 2002). The mechanism by which oxidative stress promotes apoptosis is far from understood. Possible mechanisms include increased expression of p53, a redox-sensitive transcriptional activator of several proapoptotic genes that also directly induces release of mitochondrial cytochrome *c* (CytC) through its interaction with the antiapoptotic mitochondrial protein Bcl-X_L (Chipuk *et al.*, 2003; Miller *et al.*, 2000; Soengas *et al.*, 1999). Reactive oxygen and nitrogen species can also induce the release of CytC from mitochondria through promotion of the MPT (Kowaltowski *et al.*, 2000; Borutaite *et al.*, 1999), although this event is more likely to cause necrosis due to the devastating effects of the MPT on mitochondrial energy metabolism. Oxidative alterations to mitochondrial membrane lipids or apoptotic proteins might also promote the release of CytC and other proapoptotic mitochondrial proteins through both MPT-dependent and independent mechanisms.

NEUROPROTECTION BY AVOIDING HYPEROXIA DURING CEREBRAL REPERFUSION

Intracellular conditions that exist early during reperfusion, e.g., low pH and high [Ca²⁺], can promote the generation of ROS by mitochondria and other sources (Fiskum, 1997). Microdialysis measurements demonstrate high levels of hydroxyl radical production during the first 30–45 min of reperfusion (Piantadosi and Zhang, 1996). During this same period, hyperoxia exacerbates the oxidized shift in mitochondrial redox state and delays recovery of evoked potentials compared to what is observed with normoxic animals (Feng *et al.*, 1998). Several other studies have compared hyperoxic to normoxic reperfusion using histopathology as the outcome measure. Halsey implanted O₂ electrodes in the brains of rats before subjecting them to a 20 min global ischemic insult and found a positive correlation between reoxygenation level and severity of neuronal damage (Halsey *et al.*, 1991). Gerbils treated with 100% O₂ after 15 min bilateral carotid occlusion sustained increased white matter damage (Mickel *et al.*, 1990). A study using 15 min of cardiac arrest in

dogs followed by hyperoxic resuscitation found a significant increase in the total number of injured neurons in the brain stem and spinal cord within 1 h of resuscitation (Marsala *et al.*, 1992). Our preliminary results using a 10 min canine cardiac arrest model and stereologic cell counting indicate a significant reduction in hippocampal neuronal death using normoxic compared to hyperoxic resuscitation (V. Vereczki, unpublished). In contrast, Agardh used a rat model of transient global ischemia and failed to demonstrate differences in 7 day neuronal damage after resuscitation with 100% O₂ compared to normoxia or hypoxia (Agardh *et al.*, 1991). Lipinski *et al.* also found no difference in hippocampal neuronal death 72 h following cardiac arrest in rats ventilated on 100 or 21% O₂ (Lipinski *et al.*, 1999). This model is, however, significantly different from the canine cardiac arrest model or from most human cardiac arrest scenarios as the animals experience severe hypoxia prior to cardiac arrest.

The few reported comparisons of neurologic outcome following hyperoxic and normoxic reperfusion strongly suggest that hyperoxic resuscitation is detrimental. Using a 9 min canine cardiac arrest model, Zwemer found that resuscitation with 100%-inspired O₂ resulted in worsened 12 and 24 h neurologic outcome when compared to animals receiving 21% O₂ (Zwemer *et al.*, 1994). This difference was eliminated when animals were pretreated with an antioxidant prior to the arrest and hyperoxic resuscitation. In our canine experiments using 10 min cardiac arrest, neurologic impairment measured at 24 h was significantly worse in animals ventilated on 100% O₂ during and for 1 h after resuscitation than that exhibited by dogs resuscitated on 21% O₂ and subsequently ventilated on 21–30% O₂ to maintain normal PaO₂ (Liu *et al.*, 1998). The one published negative study is the Lipinski report where no difference in 72 h neurologic impairment was observed following asphyxia-induced cardiac arrest in rats (Lipinski *et al.*, 1999). The only long-term outcome study focused on mortality and used the gerbil bilateral carotid occlusion model. Mickel and colleagues found that animals exposed to 100% O₂ for 3–6 h after 15 min global cerebral ischemia experienced a threefold increase in 14 day mortality compared with those allowed to breathe room air after ischemia (Mickel *et al.*, 1987).

MITOCHONDRIA AS SOURCES OF REACTIVE OXYGEN SPECIES

Superoxide is a normal byproduct of mitochondrial respiration and accounts for ~1% of O₂ consumed by mitochondria. Because of its extremely high reactivity and short half-life, it normally dismutates to H₂O₂ either spon-

taneously or via catalysis by mitochondrial or cytosolic superoxide dismutases. While the metabolism of H₂O₂ via peroxidases can, under some circumstances, lead to oxidative stress due to an oxidized shift in cellular redox state, the primary toxicity of elevated superoxide and H₂O₂ production is exerted by other metabolites (Fig. 1). These products include the hydroxyl radical, generated by metal-catalyzed reduction of H₂O₂, and peroxynitrite, generated by the reaction of superoxide with nitric oxide. Both of these reactive agents are capable of oxidatively modifying proteins, lipids, RNA, and DNA. As there are no known enzymatic systems for detoxifying either hydroxyl radical or peroxynitrite, endogenous interventions are limited primarily to those that reduce the production of superoxide or nitric oxide, or that promote the nontoxic metabolism of H₂O₂ to H₂O via peroxidase activities. A number of additional exogenous antioxidant approaches are available, including the use of iron chelators, spin-traps, and other natural and artificial antioxidant compounds.

While the role of mitochondrial ROS production in ischemia/reperfusion injury is often touted as important, little direct evidence is available from *in vivo* experiments. Existing evidence is based on the effects of mitochondrial respiratory inhibitors or uncouplers on markers of oxidative injury. Additional support for a critical role of mitochondrial oxidative stress in acute neuronal cell death comes from *in vitro* experiments using cultured neurons and other neural cell lines exposed to hypoxia and glucose deprivation, or to toxic levels of excitatory neurotransmitters or their agonists. From these and more recent studies, it appears that initial entry of Ca²⁺ through glutamate receptors is accumulated into mitochondria, causing an increase in mitochondrial ROS production that then causes a secondary irreversible entry of Ca²⁺ through redox-sensitive transient receptor potential (Trp) channels (Aarts *et al.*, 2003).

One controversial topic in this field is the involvement of the MPT in Ca²⁺-induced mitochondrial ROS production. MPT-mediated release of CytC can certainly stimulate mitochondrial generation of ROS by causing a reduced shift in mitochondrial redox sites associated with superoxide production. The MPT also causes a drop in mitochondrial membrane potential ($\Delta\Psi$) and a loss of mitochondrial pyridine nucleotides, both of which should depress mitochondrial generation of ROS. Recent work suggests, however, that even if mitochondrial NAD(H) were released into the cytosol in response to the MPT, the residual concentration in the mitochondrial matrix in equilibrium with the cytosolic pool could be sufficient to support substantial ROS production (Batandier *et al.*, 2004). The use of MPT inhibitors like cyclosporin A as neuroprotectants both *in vivo* and *in vitro* has met with

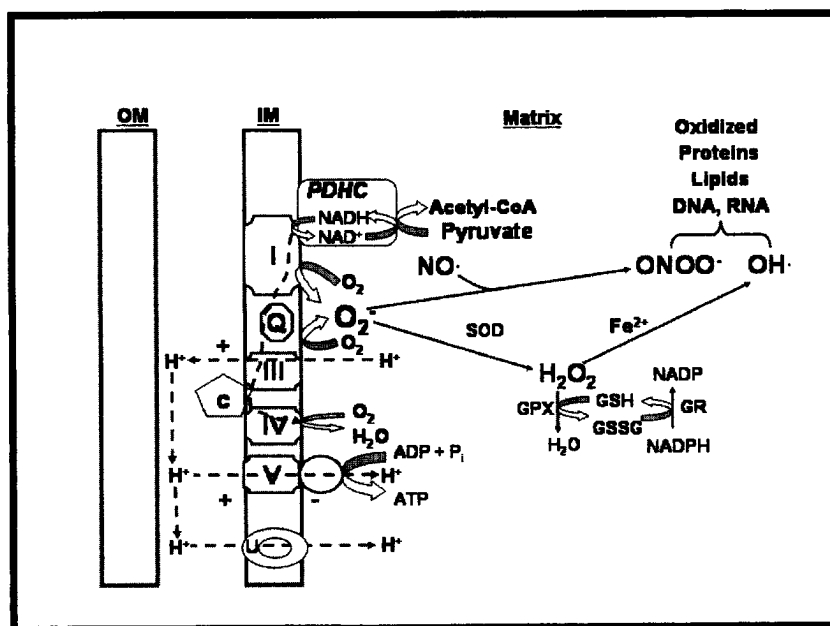


Fig. 1. Mitochondrial production and metabolism of reactive oxygen species. Superoxide (O_2^-) is produced at one or more sites within the electron transport chain and possibly via one or more matrix dehydrogenases. Superoxide is converted to hydrogen peroxide via superoxide dismutase (SOD), that is subsequently metabolized to water via the glutathione peroxidase (GPX)/glutathione reductase (GR) system. During abnormally high rates of hydrogen peroxide production and in the presence of transition metals, e.g., iron, the highly toxic hydroxyl radical is generated. Peroxynitrite can also be produced via the reaction of superoxide and nitric oxide. These metabolites can oxidize mitochondrial proteins, lipids, RNA, and DNA, contributing to oxidative stress and ultimately cell death.

mixed success (Domanska-Janik *et al.*, 2004; Kaminska *et al.*, 2001; Maciel *et al.*, 2003; Scheff and Sullivan, 1999; Uchino *et al.*, 2002). Evidence indicates that cyclosporin A is ineffective at blocking the MPT under many conditions and in certain cell types, including neurons (Fiskum *et al.*, 2003). Development of more broadly effective MPT inhibitors is therefore needed. One agent that exhibits superior inhibition of the MPT in brain mitochondria is 2-aminoethoxydiphenylborate (2-APB) (Chinopoulos *et al.*, 2003). This drug also inhibits capacitative Ca^{2+} entry associated with Trp channels and may therefore provide a multipotent approach to neuroprotection (Iwasaki *et al.*, 2001).

Several mechanism other than, or in addition to the MPT could stimulate mitochondrial ROS production during ischemia/reperfusion. Thermodynamically, any inhibition of electron flow distal to redox sites of superoxide production would promote these reactions. Thus, inhibition of electron transfer in Complex I distal to the putative iron sulfur site of superoxide generation, as occurs with rotenone, greatly stimulates ROS production with NADH-linked respiratory substrates. Inhibition at the distal points in the electron chain, as occurs with nitric oxide at Com-

plex IV, stimulates ROS production at both Complex I and at the Coenzyme Q/Complex III redox site (Brown and Borutaite, 2001). A similar situation occurs when CytC is released during apoptosis via Bax-mediated formation of pores in the outer membrane (Starkov *et al.*, 2002). When electron flow is only partially inhibited, the redox-mediated stimulation of ROS production can be counteracted by mild uncoupling, either with exogenous uncoupling agents, e.g., FCCP, or via increased expression or activity of mitochondrial uncoupling proteins. Mitochondrial ROS production is extremely sensitive to inhibition by slight depolarization and oxidized shift in redox state at high membrane potentials (Starkov and Fiskum, 2003). Thus a drop in $\Delta\Psi$ of only 15 mV reduces the rate of NADH-linked, substrate-dependent ROS formation by 50% with little effect on ATP production. Mild uncoupling may therefore constitute an effective means of immediately reducing oxidative stress in acute CNS injury paradigms (Ferranti *et al.*, 2003; Kim-Han *et al.*, 2001).

In addition to inhibiting mitochondrial superoxide production, the net production of ROS can also be reduced by promoting its detoxification to H_2O_2 and then to

H₂O. Bcl-2, an antideath protein normally thought to act by binding to outer membrane pore-forming-proapoptotic proteins, e.g., Bax, also exhibits an indirect antioxidant activity that is apparent even at the mitochondrial level (Ellerby *et al.*, 1996). We demonstrated that the inhibition of pro-oxidant-induced MPT by Bcl-2 overexpression is due to increased resistance of pyridine nucleotides to oxidation rather than a direct effect on MPT proteins (Kowaltowski *et al.*, 2000). In an attempt to explain this phenomenon, we explored the effects of Bcl-2 and other antiapoptotic Bcl-2 family members on mitochondrial bioenergetics. Through careful calibration techniques, we found that mitochondria from Bcl-2-overexpressing cells do not exhibit higher membrane potential, in contrast to previous reports indicating a difference in $\Delta\Psi$. In agreement with other investigators, we found that overexpression of Bcl-2 was associated with an increase in basal mitochondrial ROS (H₂O₂) production. This counterintuitive phenotype was also observed in cells overexpressing Bcl-X_L and Mcl-1, two additional cytoprotective Bcl-2 family members (A. Kowaltowski, unpublished). Most importantly, when the overexpressing cells were treated for 48 h with low levels of uncoupler that eliminate the elevated level of mitochondrial ROS production, the cells lose their abnormally high peroxidase activity and their resistance to acute necrotic cell death caused by exposure to high concentrations of exogenous H₂O₂. It therefore appears that the antioxidant activity of at least three antiapoptotic Bcl-2 family members is similar to preconditioning paradigms where sublethal levels of stress cause up-regulation of proteins that protect against normally lethal levels of stressful stimuli. While overexpression of Bcl-2 increases basal ROS production, this effect stimulates the expression of one or more antioxidant enzymes resulting in a net resistance to oxidative stress.

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Mitochondrial Uncoupling as a Therapeutic Target Following Neuronal Injury

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Mitochondrial dysfunction is a prominent feature of excitotoxic insult and mitochondria are known to play a pivotal role in neuronal cell survival and death following injury. Following neuronal injury there is a well-documented increase in cytosolic Ca^{2+} , reactive oxygen species (ROS) production and oxidative damage. In vitro studies have demonstrated these events are dependent on mitochondrial Ca^{2+} cycling and that a reduction in membrane potential is sufficient to reduce excitotoxic cell death. This concept has gained additional support from experiments demonstrating that the overexpression of endogenous mitochondrial uncoupling proteins (UCP), which decrease the mitochondrial membrane potential, decreases cell death following oxidative stress. Our group has demonstrated that upregulation of UCP activity can reduce excitotoxic-mediated ROS production and cell death whereas a reduction in UCP levels increases susceptibility to neuronal injury. These findings raise the possibility that mitochondrial uncoupling could be a potential novel treatment for acute CNS injuries.

KEY WORDS: Neuronal cell death; traumatic brain injury; excitotoxicity; spinal cord injury; reactive oxygen species.

Central Nervous System (CNS) trauma results in several pathophysiological events that contribute to neuronal damage and death, including glutamate-mediated excitotoxicity and the formation of reactive oxygen species (ROS) (Azbill *et al.*, 1997; Braughler and Hall, 1992; Faden *et al.*, 1989; Sullivan *et al.*, 1998, 1999a). The ensuing loss of neuronal tissue is believed to evolve in a biphasic manner consisting of the primary mechanical insult and a progressive secondary necrosis (Cooper, 1985; Faden, 1993; Siesjo *et al.*, 1995). Alterations in excitatory amino acids (EAA), increased oxidative stress (ROS), and the disruption of Ca^{2+} homeostasis are major factors contributing to the ensuing neuropathology (Braughler *et al.*, 1985; Braughler and Hall, 1992; Choi *et al.*, 1990; Faden *et al.*, 1989). Compelling experimental data also demonstrates that mitochondria play a fundamental role in the death cascade, and mitochondria

have been directly linked to EAA-mediated neurotoxicity (Brustovetsky *et al.*, 2002; Jiang *et al.*, 2001; Nicholls and Budd, 1998a; Stout *et al.*, 1998; Sullivan *et al.*, 2003). The present studies are based on the hypothesis that injury-induced glutamate release increases mitochondrial Ca^{2+} cycling/overload ultimately leading to mitochondrial dysfunction and that transient mitochondrial uncoupling can confer neuroprotection following traumatic brain injuries (TBI) and spinal cord injuries (SCI).

Extrinsic mitochondrial uncouplers are compounds that facilitate the movement of protons from the mitochondrial inner-membrane space into the mitochondrial matrix. Intrinsic uncoupling can be mediated via the activation of endogenous mitochondrial uncoupling proteins (UCP) which utilize free fatty acids to translocate protons. This short circuit "uncouples" the pumping of protons out of the matrix via the electron transport system (ETS) from the flow of protons through the ATP synthase and results in a coincidental reduction in the mitochondrial membrane potential ($\Delta\Psi$). While it is obvious that long-term, complete uncoupling of mitochondria would be detrimental, a transient or "mild uncoupling" could confer neuroprotection. Mild uncoupling during the acute phases

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of injured-induced excitotoxicity would be expected to reduce mitochondrial Ca^{2+} uptake (cycling) and ROS production, since both are $\Delta\Psi$ -dependent.

Following TBI and SCI, there is a significant loss of mitochondrial homeostasis, resulting in increased mitochondrial ROS production and disruption of synaptic homeostasis (Azbill *et al.*, 1997; Sullivan *et al.*, 1998, 1999a,b; Xiong *et al.*, 1997). This implicates an underlying pivotal role for mitochondria in the sequelae of injury-related neuropathology. Our laboratories and others have solidified this theory by demonstrating that therapeutic intervention with cyclosporin A following experimental TBI significantly reduces mitochondrial dysfunction (Sullivan *et al.*, 1999a) and cortical damage (Scheff and Sullivan, 1999; Sullivan *et al.*, 2000a,c), as well as cytoskeletal changes and axonal dysfunction (Okonkwo *et al.*, 1999; Okonkwo and Povlishock, 1999). At least part of the mechanism by which CsA affords neuroprotection is via the maintenance of mitochondrial homeostasis by inhibiting the opening of the mitochondrial permeability transition pore (Buki *et al.*, 1999; Okonkwo *et al.*, 1999; Okonkwo and Povlishock, 1999; Scheff and Sullivan, 1999; Sullivan *et al.*, 1999a). Furthermore, maintaining mitochondrial bioenergetics by dietary supplementation with creatine has also proved effective in ameliorating neuronal cell death and reduces mitochondrial ROS production and maintaining ATP levels following TBI (Sullivan *et al.*, 2000b).

Although the complex mechanisms of secondary neuronal injury are poorly understood, it is clear that EAA neurotoxicity plays an important role (Rothman and Olney, 1995). This results in excessive entry of Ca^{2+} , leading to a loss of cellular homeostasis and subsequent neuronal Ca^{2+} overload. Ca^{2+} is the most common signal transduction element in cells, but unlike other second-messenger molecules, it is required for life. Paradoxically, prolonged high levels of $[\text{Ca}^{2+}]_i$ leads to cell death (Choi, 1992). Since Ca^{2+} cannot be metabolized like other second-messenger molecules, it must be tightly regulated by cells. Numerous intracellular proteins and some organelles have adapted to bind or sequester Ca^{2+} to ensure that homeostasis is maintained. *Mitochondria are one such organelle* (Ichas and Mazat, 1998; Rizzuto *et al.*, 1999, 2000). During excitotoxic insult, Ca^{2+} uptake into mitochondria has been shown to increase ROS production, inhibit ATP synthesis and induce mitochondrial permeability transitions (Brustovetsky *et al.*, 2002; Dugan *et al.*, 1995; Reynolds and Hastings, 1995; Sengpiel *et al.*, 1998; White and Reynolds, 1996). It is also important to note that inhibition of mitochondrial Ca^{2+} uptake by reducing $\Delta\Psi$ (chemical uncoupling) following excitotoxic insults is neuroprotective, emphasizing the

pivotal role of mitochondrial Ca^{2+} uptake in EAA neuronal cell death (Nicholls and Budd, 1998a,b; Stout *et al.*, 1998).

Free radical production is a byproduct of ATP generation in mitochondria via the electron transport chain. Electrons escape from the chain and reduce O_2 to O_2^- . Normally cells convert O_2^- to H_2O_2 utilizing both manganese superoxide dismutase, which is localized to the mitochondria, and copper-zinc superoxide dismutase found in the cytosol. H_2O_2 is rapidly converted to H_2O via catalase and glutathione peroxidase, but has the potential to be converted to the highly reactive hydroxyl radical (OH^\cdot) via the Fenton reaction, underlying ROS neurotoxicity. OH^\cdot rapidly attacks unsaturated fatty acids in membranes causing lipid peroxidation and the production of 4-hydroxynonenal (HNE) that conjugates to membrane proteins, impairing their function (Azbill *et al.*, 1997; Keller *et al.*, 1997a,b; Mark *et al.*, 1997; Sullivan *et al.*, 1998). In particular, ROS induction of lipid peroxidation and protein oxidation products may be particularly important in neurodegeneration (for review see Mattson, 1998) and TBI (Braugher *et al.*, 1985; Braugher and Hall, 1989, 1992; Sullivan *et al.*, 1998).

Mitochondrial ROS production is intimately linked to $\Delta\Psi$ such that hyperpolarization (high $\Delta\Psi$) increases and promotes ROS production (Liu *et al.*, 2002; Skulachev, 1996, 1998; Starkov *et al.*, 2002; Starkov and Fiskum, 2003; Votyakova and Reynolds, 2001). Since the magnitude of ROS production is largely dependent on—and correlates with— $\Delta\Psi$ even a modest reduction via increased proton conductance (decreases $\Delta\Psi$, the electrochemical proton gradient) across the mitochondrial inner membrane (uncoupling) reduces ROS formation (Kim-Han *et al.*, 2001; Skulachev, 1996; Sullivan *et al.*, 2003, in press-b; Votyakova and Reynolds, 2001).

Endogenous mitochondrial uncoupling is mediated by members of the UCP family, which function to dissociate ATP production from oxygen consumption in mitochondria of muscle and fat tissues (Nicholls and Ward, 2000), leading to heat generation. UCPs are activated by FFAs, superoxide and inhibited by purine nucleotides (Echtay *et al.*, 2002) (also see Argiles *et al.*, 2002; Harper *et al.*, 2001; Zackova and Jezek, 2002 for review). Five mitochondrial UCPs exist in the human genome and among characterized uncoupling proteins, UCP2, UCP4, and UCP5/BMCP1 have recently been shown to be significantly expressed in the CNS (Arsenijevic *et al.*, 2000; Diano *et al.*, 2000; Horvath *et al.*, 1999; Kim-Han *et al.*, 2001). However, unlike UCP1 that is present only in brown adipose tissue and used to generate heat in cold environments (i.e., thermogenesis), their physiological role(s) are unclear.

Several hypotheses have been put forth concerning possible physiological roles of the UCPs including energy partitioning, energy balance, and control of metabolism which may be pivotal in obesity and diabetes (for review see Argiles *et al.*, 2002; Jezek, 2002). Skulachev was the first to hypothesize that mild uncoupling could be beneficial since it causes a decrease in ROS production (Skulachev, 1996; and preceding section). Several studies have now demonstrated roles for UCPs in modulating ROS production. UCP2 (Arsenijevic *et al.*, 2000) or UCP3 (Vidal-Puig *et al.*, 2000) knockout mice exhibit increased ROS in macrophages and muscle, respectively. Leptin-deficient mice have decreased levels of UCP2 and increased ROS production in macrophages (Lee *et al.*, 1999). Overexpression of UCP2 (Li *et al.*, 2001) or UCP5/BMCP1 (Kim-Han *et al.*, 2001) has also been shown to decrease cell death following H_2O_2 exposure and ROS production, respectively. UCP2 overexpression has also been demonstrated to reduce ROS production and increase tissue sparing in vivo following ischemia or TBI (Mattiasson *et al.*, 2003).

On the basis of these initial reports, it is reasonable that increasing UCP activity by modulating dietary fat could directly modulate and reduce mitochondrial ROS production and subsequent oxidative damage. We have indeed shown that the converse (i.e., reducing dietary fat in immature animals) does rapidly reduce neuronal UCP expression/activity and increases mitochondrial ROS production. These changes in mitochondrial UCP activity and ROS production decrease the resistance of these immature animals to excitotoxic insult resulting in increased neuronal cell death following seizure activity, implicating a neuroprotective role for UCP2 and mitochondrial uncoupling in neuronal injury (Sullivan *et al.*, 2003). These data also suggest that increasing dietary fat content would increase UCP activity and reduce ROS production, both of which we have recently demonstrated to occur in vivo (Sullivan *et al.*, in press-a).

Several studies have demonstrated that mitochondrial uncoupling in vitro reduces neuronal mitochondrial Ca^{2+} loading and can inhibit excitotoxic cell death (Billups and Forsythe, 2002; Nicholls and Budd, 1998a,b; Pivovarova *et al.*, 2002; Stout *et al.*, 1998). To date only one study has assessed the potential for using mitochondrial uncouplers (2,4-DNP) as neuroprotective agents in an in vivo model of excitotoxicity (Maragos *et al.*, 2003). Since it is well-established that excitotoxicity is a major player in TBI- and SCI-induced neuronal cell death and results in significant mitochondrial dysfunction, we designed several experiments to test the hypothesis that the mitochondrial uncouplers 2,4-DNP and FCCP would be neuroprotective following TBI and SCI.

The results demonstrate that rats administered a mitochondrial uncoupler have less tissue loss, improved behavioral outcomes and demonstrate a reduction in mitochondrial oxidative damage, Ca^{2+} loading, and dysfunction following SCI or TBI. The results also demonstrate that mitochondrial uncouplers significantly reduces mitochondrial dysfunction associated with injury whereas a 2,4-DNP analogue 2,4,6-trinitrophenol (TNP), which lacks the ability to uncouple intact mitochondria, did not provide any neuroprotection. Importantly, postinjury fasting of animals (24 h) following TBI yields similar results, perhaps by utilizing endogenous mitochondrial uncoupling proteins (UCP). Together these results implicate important mitochondrial events that could be potential novel interventions and novel targets for the treatment of TBI and SCI as well as other acute neuronal injuries.

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Regulation of Synaptic Transmission by Mitochondrial Ion Channels

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Mitochondria are abundant within neuronal presynaptic terminals, where they provide energy for sustained neurotransmitter secretion. Injection of Bcl-xL protein into squid giant presynaptic terminal potentiates neurotransmitter release, while a naturally occurring, proteolytic fragment of BCL-xL causes rundown of synaptic function. The cleaved form of BCL-xL generates large, multiconductance ion channel activity in synaptic mitochondrial outer membranes. A rapid onset of synaptic rundown can also be produced by depriving the synapse of oxygen, and hypoxia also induces large channel activity in mitochondrial outer membranes. Channel activity induced by cleaved BCL-xL or by hypoxia is attenuated by NADH, an inhibitor of the voltage-dependent anion channel (VDAC) of mitochondrial outer membranes. Finally, the large conductances elicited by hypoxia are prevented by the addition of a protease inhibitor that prevents cleavage of BCL-xL. The opposing activities of BCL-xL and its proteolytic fragment may regulate the release of ATP from mitochondria during synaptic transmission.

KEY WORDS: Neurotransmission; synapse; ischemia; mitochondria; VDAC; BCL-xL.

Regulation of events at the presynaptic terminal of a synapse is important for determining whether a neuronal pathway will become strengthened during such processes as learning and the making of new memories. Conversely, biochemical events at the synapse can cause the synapse to fail during neurodegeneration, such as in Alzheimer's disease, or in acute injury, such as during brain ischemia. Studies of the modulation of synaptic transmission comprise an important area of focus in the field of Neurobiology.

The squid giant presynaptic terminal is a well-established model system for studying neurotransmission. It has an extremely large (1 mm) presynaptic terminal that enables investigators to study synaptic properties with relative ease. Electron micrographs reveal collections of synaptic vesicles adjacent to the area of contact between pre- and postsynaptic cells (Jonas *et al.*, 1999; Martin and Miledi, 1975). Deeper inside the terminal are arrays of neurofilaments and numerous mitochondria that are thought to provide the energy for neurotransmission

and to manage the calcium that enters at the active zone. Most other types of organelles are absent. In mammalian synapses, presynaptic mitochondria have specific morphological features that differentiate them from other types of mitochondria (Tolbert and Morest, 1982). For example, some presynaptic mitochondria are tethered to active zones and physically linked to chains of vesicles (Rowland *et al.*, 2000).

Previous studies have begun to shed light on the role of mitochondria in the synapse, and have hinted that, rather than simply making ATP constitutively to provide the energy for synaptic activity, they regulate the supply of ATP. Mitochondria are also known to buffer cytoplasmic calcium ions. Sustained elevations in presynaptic calcium following rapid, repetitive neuronal firing are not only correlated with enhancement of synaptic transmission (Swandulla *et al.*, 1991; Wang and Kaczmarek, 1998), but also require intact mitochondria in secretory cells (Babcock and Hille, 1998) and in neurons (Billups and Forsyth, 2002; Friel and Tsien, 1994; Nguyen *et al.*, 1997; Tang and Zucker, 1997). Nevertheless, the specific molecular mechanisms that define the role of mitochondria in calcium and metabolite management during high frequency presynaptic activity are not yet known.

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INTRACELLULAR ION CHANNEL RECORDING TECHNIQUE

The activity of mitochondrial ion channels is required for calcium buffering and release of energy metabolites. To characterize channels on mitochondrial membranes that might be important during synaptic transmission and during apoptosis, we developed a technique to record from intracellular ion channels in intact cells (Jonas *et al.*, 1997, 1999). The intracellular organelle recording technique is a variant of the patch clamp technique but the patch electrode is contained within an outer, large bore microelectrode. The concentric electrodes can be manipulated past the plasma membrane, after which the outer electrode is withdrawn, exposing the inner tip. Negative pressure causes the inner tip to form a high resistance seal on intracellular membranes, after which single channel data is gathered either on the organelle, or after excision of the patch into the cytoplasm or bath. Lipophilic fluorescent dyes (Pagano *et al.*, 1989) have been included in the patch pipette, and give information about the intracellular location of the pipette tip. In the squid presynaptic terminal, as in many other presynaptic terminals, mitochondria are the only internal organelles that are compatible with seal formation by the patch pipettes, which have tip diameters of approximately ~180–200 nm by scanning electron microscopy.

EFFECTS OF SYNAPTIC STIMULATION ON MITOCHONDRIAL ION CHANNEL ACTIVITY

Mitochondrial recordings inside the resting squid presynaptic terminal reveal small conductance activity. Very infrequently, much larger conductances occur spontaneously. Electrical stimulation of the squid presynaptic terminal to evoke synaptic transmission, however, causes a marked change in activity and conductance of mitochondrial patches. During a brief, high frequency, train of stimuli, mitochondrial ion channel activity increases inside the terminals, resulting in an approximately 60-fold enhancement of membrane conductance lasting up to 60 s (Jonas *et al.*, 1999). Activity then gradually decreases in frequency and amplitude over the 5–30 s following the period of enhanced activity.

The delayed onset and the persistence of the mitochondrial channel activity after stimulation implies that this increased activity on the mitochondrial outer membrane is not simultaneous with the opening of plasma membrane channels, and suggests that this increase may depend on an intracellular second messenger. During synaptic stimulation, there is a build-up of calcium in

the presynaptic terminal. This is thought to be responsible for a form of short-term synaptic plasticity termed posttetanic potentiation, and this persistent calcium elevation has been found previously to depend on mitochondria (Friel and Tsien, 1994; Tang and Zucker, 1997). In recordings from mitochondrial membranes inside the terminal in a calcium-deficient bathing medium, there is no response to stimulation of the presynaptic nerve, demonstrating that the evoked intracellular membrane channel activity is dependent on calcium influx. Intracellular membrane channel activity is also dependent on an intact mitochondrial membrane potential. Uncoupling mitochondria with FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) completely eliminates the increase in conductance during stimulation. FCCP also eliminates posttetanic potentiation. The timing of the changes in mitochondrial conductance and their dependence on calcium suggest that opening of a mitochondrial channel is important for short-term plasticity of the synapse.

The identities of the mitochondrial channels responsible for changes during synaptic transmission are not yet known. The channels of interest that may undergo regulation during synaptic events include those of both the inner and outer membranes. The calcium-selective uniporter (Kirichok *et al.*, 2004), and the calcium-sensitive permeability transition pore (Bernardi, 1996) are candidates for inner membrane channels. Once calcium and metabolites are released from the matrix into the intermembrane space, they are released across the outer membrane to reach the cytosol. The voltage-dependent anion channel (VDAC), which is a ubiquitous protein in mitochondrial outer membrane (Colombini *et al.*, 1996) may perform this function. More recently, channels formed by interactions of BCL-2 family proteins with mitochondrial membranes (Kroemer, 1997; Reed, 1997) have been found to release or inhibit the release of mitochondrial components such as cytochrome *c* into the cytosol. Many BCL-2 family proteins, such as BCL-xL, are endogenously present in mitochondrial membranes (Kaufmann *et al.*, 2003). BCL-xL is a potent inhibitor of programmed cell death and is abundantly expressed in neurons of the adult brain (Blömer *et al.*, 1998; Boise *et al.*, 1993; Frankowski *et al.*, 1995; González-García *et al.*, 1995; Krajewski *et al.*, 1994) where its role in developmental apoptosis is obviously constrained. It has been suggested that the role of BCL-xL in adult neurons is to protect cells from death by regulating export of ATP from mitochondria and/or by blocking the activation of proapoptotic proteins (Bañez *et al.*, 2002; Vander Heiden *et al.*, 2000; Zong *et al.*, 2001), but other roles for this important molecule have yet to be elucidated.

Although BCL-2 family proteins are able to conduct ions when reconstituted into artificial lipid bilayers (Antonsson *et al.*, 1997; Minn *et al.*, 1997; Schendel *et al.*, 1998; Schlesinger *et al.*, 1997), the precise biochemical mechanisms by which they regulate mitochondrial permeability and apoptosis in cells or whether they form channels *in vivo* was not previously known.

BCL-xL in the Presynaptic Terminal of the Squid Giant Synapse Enhances Synaptic Transmission and Induces Channel Activity in Mitochondria

BCL-xL is present on mitochondria in the stellate ganglion of the adult squid (Jonas *et al.*, 2003), where it could play a role in protection against acute insults to the nervous system. To determine the action of BCL-xL on mitochondrial membrane conductances, we recorded from mitochondria inside the synapse with recombinant BCL-xL in the patch pipette solution (Jonas *et al.*, 2003). Activity with multiple conductances was readily detected in these mitochondrial patches (Fig. 1). Activity typically switched between different conductance levels every few seconds, but a single conductance level could also occasionally be maintained for several minutes. Recordings made with BCL-xL in the patch pipette demonstrated larger conductances than those observed in control recordings.

Activity on mitochondrial membranes during synaptic transmission could be a consequence of, or an integral link in the chain of events that leads to posttetanic potentiation. Because BCL-xL produces a change in mitochondrial membrane conductance, it is a possible candidate for a mitochondrial membrane channel that could alter

synaptic transmission. Indeed, injection of BCL-xL into presynaptic terminals enhances the rate of rise of postsynaptic responses in both healthy synapses and in those in which transmission has run down to the point that the postsynaptic potential no longer triggers postsynaptic action potentials.

ATP Enhances Synaptic Transmission

Work with nonneuronal cells has suggested that BCL-xL regulates the flux of metabolites across the outer mitochondrial membrane to facilitate transport of ATP into the cytosol following a death stimulus (Vander Heiden *et al.*, 2000, 2001). Consistent with these findings, in squid synaptic terminals, direct microinjection of ATP into the presynaptic cell effectively enhances the postsynaptic responses, and injected ATP occludes the effects of BCL-xL. The findings raise the possibility that BCL-xL may enhance synaptic activity by triggering release of ATP from mitochondria, and support the idea that the ion channel function of antiapoptotic proteins may include regulation of the release of ATP. The moderate size of the conductance (200–500 pS) produced by the antiapoptotic protein in mitochondrial membranes suggests that it would be unable to release large components of mitochondria such as cytochrome *c* during apoptosis.

INDUCTION OF LARGE CONDUCTANCES DURING INSULTS TO THE SYNAPSE

In apoptosis or in insults to the nervous system, a different set of changes occur in the outer membrane. These are associated with the release of cytochrome *c* and

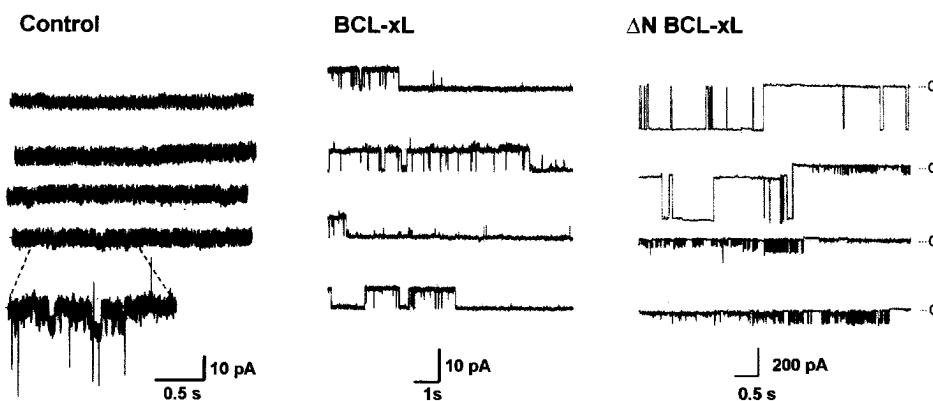


Fig. 1. Different forms of multiconductance channel activity are produced by the antiapoptotic protein BCL-xL and its proapoptotic cleavage fragment ΔN BCL-xL applied to mitochondria within the presynaptic terminal of the squid. Left panel shows small conductance channel activity in control mitochondria.

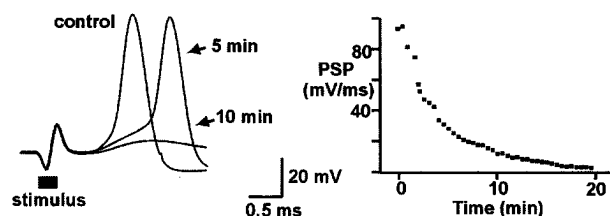


Fig. 2. Hypoxia causes synaptic rundown. Postsynaptic responses are shown at different times after the onset of oxygen deprivation. The second panel shows the change in slope of postsynaptic potentials over time after the onset of oxygen deprivation.

other factors from the intermembrane space (Gross *et al.*, 1999). Under these conditions BCL-2 family proteins, either by interaction with VDAC or by independent mechanisms, may also contribute to channel activity in the outer membrane without any activation of the inner membrane (Polster *et al.*, 2001). In order to study how mitochondria participate in pathological states of the synapse that might be similar to apoptotic conditions, and how the outer membrane channels VDAC and BCL-xL participate in these events, we studied the reverse of synaptic potentiation, synaptic rundown during hypoxia.

Hypoxia Induces Multiconductance Channel Activity in Synaptic Mitochondria

We used the giant synapse of the squid stellate ganglion as a model system to study the effects of hypoxia on mitochondrial ion channel activity. The presynaptic terminal of this synapse is very sensitive to hypoxia, which attenuates synaptic transmission over 10–30 min (Fig. 2). Hypoxia can be induced in squid giant stellate ganglia by eliminating perfusion of oxygenated sea water. Patch clamp recordings of channel activity on mitochondria during hypoxia show that, in contrast to controls, a new, large multiconductance channel appears on average within about 13 min after the start of hypoxia. Conductances ranging from 300 pS to 2.0 nS can be detected in these hypoxic neurons using invertebrate intracellular solution.

The channel activity produced by hypoxic conditions is much larger than that observed with application of full length BCL-xL to the patch, but closely resembles that produced by applying a proapoptotic version of BCL-xL to the mitochondrial membranes. Whether BCL-xL, which we have shown to be correlated with potentiation of the synapse, could also be responsible for synaptic rundown during hypoxia, is an interesting question. BCL-xL protein can be cleaved between the BH4 and BH3 domains by zVAD-sensitive proteases caspase-3 (Asp61, Asp76) and

calpain (Ala60) to produce a proapoptotic C-terminal fragment, Δ N BCL-xL lacking amino acids 2–76 (Clem *et al.*, 1998; Fujita *et al.*, 1998; Nakagawa and Yuan, 2000). In the squid presynaptic terminal, hypoxia produces proteolysis of BCL-xL, an effect that can be blocked by the protease inhibitor zVAD.

In mammalian cells, overexpression of Δ N BCL-xL potently induces loss of mitochondrial membrane potential, cytochrome *c* release from mitochondria and apoptosis (Basanez *et al.*, 2001; Kirsch *et al.*, 1999). Injection of Δ N BCL-xL into the squid presynaptic terminal has the opposite effect from that of full length BCL-xL, it attenuates synaptic transmission (Jonas *et al.*, 2003). In addition, hypoxic rundown has the same time course as that induced by Δ N BCL-xL and application of Δ N BCL-xL to mitochondrial membranes in intact terminals produces large multiconductance channel activity, similar to that seen during hypoxia. This activity is dependent on an intact BH3 domain and on interaction with mitochondrial membranes. Peak conductances in different recordings range between 300 pS and 3.8 nS, similar to channel activity observed during hypoxia. The large conductance of this channel could be responsible for the release of cytochrome *c* and other proapoptotic factors into the cytosol during hypoxic or degenerative synaptic rundown.

Formation of Δ N BCL-xL Mitochondrial Channels Requires VDAC

Because the gigohm seals were formed directly on intracellular organelles within the presynaptic terminal, it is likely that the membrane contacted by the patch pipette is the outer mitochondrial membrane. The conductance of this membrane is known to be reduced by millimolar concentrations of NADH (Lee *et al.*, 1994; Wunder and Colombini, 1991). In lipid bilayers, NADH has also been shown to reduce the conductance of VDAC, a relatively nonselective channel that is believed to be the major conductance pathway across the outer membrane. In squid, NADH specifically reduces the probability of large conductance activity induced by Δ N BCL-xL or hypoxia in mitochondrial membranes. It fails, however, to inhibit the actions of Δ N BCL-xL on the permeability of artificial lipid membranes. Taken together, the data suggest that NADH alters the conductance of hypoxia- or Δ N BCL-xL-induced channels by acting on a mitochondrial component other than the BCL-xL protein alone.

To further investigate the hypothesis that Δ N BCL-xL or hypoxia require VDAC to form channels, recordings were made on mitochondrial membranes prepared

from wild type yeast and from yeast that lack the *por1* gene, which encodes the VDAC-1 channel (YVDAC1) (Lee *et al.*, 1998; Lohret and Kinnally, 1995).

Recording from the outer membrane of isolated wild type yeast mitochondria revealed a voltage-dependent behavior with properties similar to those previously described for VDAC in artificial membranes (Colombini *et al.*, 1996). In a separate set of recordings on these wild type mitochondria, the inclusion of Δ N BCL-xL protein in the pipette solution resulted in a very different pattern of activity. The typical VDAC-like activity could no longer be detected, and significantly larger conductance activity was detected at both positive and negative potentials. These large conductances were similar to those observed after the addition of Δ N BCL-xL in squid presynaptic terminal, and were also markedly attenuated by the addition of NADH.

Large conductance activity could be recorded under control conditions in mitochondrial membranes from mutant Δ POR1 yeast lacking the VDAC channel, but, in contrast to the wild type mitochondria, this activity was completely unaffected by Δ N BCL-xL and was not inhibited by NADH, suggesting that VDAC is required for formation of channels by the Δ N BCL-xL protein.

In summary, by using techniques that allow mitochondrial membrane recordings within living cells, we can begin to describe mitochondrial ion channel activities that play a role in synaptic transmission.

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Mitochondrial Dysfunction Contributes to Cell Death Following Traumatic Brain Injury in Adult and Immature Animals

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Secondary injury following traumatic brain injury (TBI) is characterized by a variety of pathophysiologic cascades. Many of these cascades can have significant detrimental effects on cerebral mitochondria. These include exposure of neurons to excitotoxic levels of excitatory neurotransmitters with intracellular calcium influx, generation of reactive oxygen species, and production of peptides that participate in apoptotic cell death. Both experimental and clinical TBI studies have documented mitochondrial dysfunction, and animal studies suggest this dysfunction begins early and may persist for days following injury. Furthermore, interventions targeting mitochondrial mechanisms have shown neuroprotection after TBI. Continued evaluation and understanding of mitochondrial mechanisms contributing to neuronal cell death and survival after TBI is indicated. In addition, important underlying factors, such as brain maturation, that influence mitochondrial function should be studied. The ability to identify, target, and manipulate mitochondrial dysfunction may lead to the development of novel therapies for the treatment of adult and pediatric TBI.

KEY WORDS: Brain mitochondria; development; pediatric; cytochrome *c*; bcl-2; membrane permeability transition; apoptosis.

Approximately 1.5 million people sustain traumatic brain injury (TBI) in the United States each year (CDC, 1999). Of these, over 50,000 patients die annually, accounting for greater than one-third of all injury-related deaths (Sosin *et al.*, 1995). Among survivors, almost one-fourth million people sustain a degree of injury that warrants hospitalization. In 1995, it was estimated that the total direct and indirect financial costs of TBI-related injuries were greater than \$50 billion (Thurman, 2001). The long-term social and emotional burden may be even greater, with an estimated 5.3 million men, women, and children living with a TBI-related disability (CDC, 1999). Despite the significant public health impact of TBI, limited neuroprotective interventions exist for those suffering severe TBI.

MITOCHONDRIA AND TBI – PRECLINICAL STUDIES

Growing evidence suggests an important role for mitochondria as subcellular targets for neuroprotection after TBI. Factors that both inhibit and promote neuronal apoptosis appear to work by influencing mitochondrial cytochrome *c* release, and pathways that promote necrotic cell death, such as excitotoxicity and oxidative stress, have profound influences on mitochondrial function (Fig. 1). Importantly, both preclinical and clinical studies have documented apoptotic and necrotic neural cell death occurring after TBI, and more recent studies have begun to define the significant influence of mitochondrial dysfunction on these cell death pathways.

Although the importance of mitochondria following brain injury has been suggested and studied for many years (reviewed in Fiskum *et al.*, 1999), the majority of studies describing mitochondrial roles specifically in TBI have been conducted in the last decade. Early studies evaluated changes in mitochondrial respiration in the first few hours

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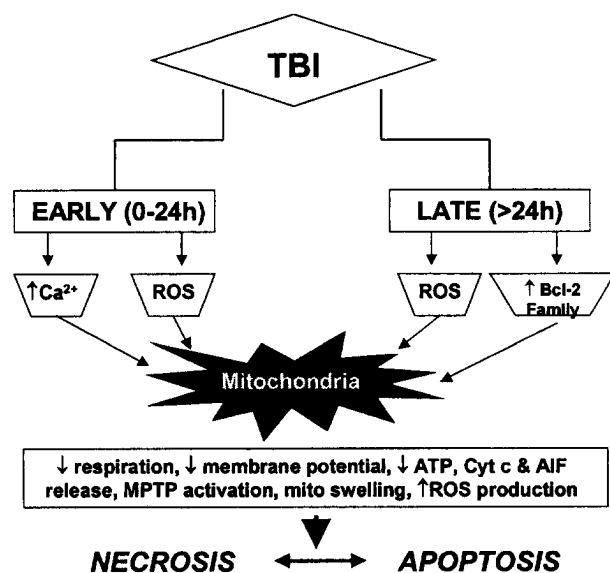


Fig. 1. Mechanisms of mitochondrial injury after TBI. TBI results in both early and delayed neural injury, both of which can have a profound influence on mitochondrial function and ultimately lead to necrotic and apoptotic cell death.

after injury and found slight reductions in the active, phosphorylating rate of mitochondrial respiration 4 h after fluid percussion TBI (Vink *et al.*, 1990). Subsequent studies using the controlled cortical impact model of TBI defined more dramatic alterations in mitochondrial respiration that began within 1 h of injury and persisted for at least 14 days (Xiong *et al.*, 1997a). In addition, mitochondria isolated from the hemisphere ipsilateral to injury demonstrated reduced ability to sequester Ca^{2+} (Xiong *et al.*, 1997a). These alterations in mitochondrial respiration and Ca^{2+} transport were reversible by postinjury treatment with the calcium channel blocker, SNX-111 (Verweij *et al.*, 1997) and the antioxidant, U-101033E (Xiong *et al.*, 1997b), both alone and in combination (Xiong *et al.*, 1998). These studies emphasize the potential for neuroprotection after TBI through pharmacologic intervention that directly targets mitochondrial dysfunction.

In addition to perturbations in mitochondrial respiration and Ca^{2+} homeostasis, TBI has recently been shown to have effects on mitochondrial membrane potential. Isolated mitochondria and synaptosomes from injured cortex show reduced membrane potential and evidence of mitochondrial inner membrane permeability changes (Sullivan *et al.*, 1999). Membrane potential was restored by the administration of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore (MPTP). Similar types of mitochondrial abnormalities involving alterations in membrane potential with resultant mitochondrial

swelling have also been seen in models of TBI examining axonal injury (Pettus and Povlishock, 1996). This series of investigations has described a key role for loss of mitochondrial integrity in the secondary axotomy that occurs following TBI (Buki *et al.*, 1999; Okondkwo *et al.*, 1999; Okonkwo and Povlishock, 1999).

Another important pathway with great significance in TBI is mitochondrial cytochrome *c* release and resultant neuronal apoptosis (reviewed in Raghupathi *et al.*, 2000). Experimental studies of TBI have demonstrated mitochondrial cytochrome *c* release in many models of TBI, including cold injury-induced brain trauma (Morita-Fujimura *et al.*, 1999), traumatic axonal injury (Buki *et al.*, 2000), and controlled cortical impact (Lewen *et al.*, 2001; Sullivan *et al.*, 2002). Downstream events, such as caspase activation, have also been well documented in animal models of TBI (Clark *et al.*, 2000; Keane *et al.*, 2001; Knobloch *et al.*, 2002; Sullivan *et al.*, 2002; Yakovlev *et al.*, 1997).

MITOCHONDRIA AND TBI – CLINICAL STUDIES

Although the number of studies directly evaluating mitochondrial function after TBI in humans is limited, they have generally supported findings seen in animal models of TBI. Brain mitochondria isolated from human victims of TBI have shown impaired rates of respiration and ATP synthesis (Verweij *et al.*, 1997, 2000). One interesting study of human autopsy tissue compared brain mitochondrial DNA deletions in short-term survivors of cardiac arrest, long-term survivors of TBI and age-matched controls, evaluating the role of mitochondrial gene expression in brain injury (McDonald *et al.*, 1999). They discovered a significantly lower incidence of mitochondrial DNA deletions in long-term survivors of TBI and hypothesized that chronic free radical-induced mitochondrial DNA damage may ultimately influence the survival of head-injury victims.

A few clinical studies have demonstrated evidence for apoptotic cell death involving mitochondrial pathways after TBI in adults and children. For example, caspase activation was documented in human TBI tissue (Clark *et al.*, 1999), and bcl-2 protein was increased in brain tissue from adult patients and in cerebrospinal fluid (CSF) from pediatric patients after TBI (Clark *et al.*, 1999, 2000). Importantly, bcl-2 CSF concentration correlated with patient survival, suggesting a neuroprotective role for bcl-2 in pediatric TBI victims. Very recent clinical studies in pediatric TBI have discovered elevations in two mitochondrial proteins, heat shock protein 60 (Hsp60) and

cytochrome *c*, in the CSF of head-injured children compared to noninjured pediatric controls (Lai *et al.*, 2003; Strange *et al.*, 2003). In these studies Hsp60 correlated with injury severity and cytochrome *c* levels correlated with child abuse victims and female gender. The presence of these integral mitochondrial proteins in the CSF suggests the presence of mitochondrial damage in these patients, and the correlation with specific injury and demographic features may prove to be helpful in defining subpopulations likely to respond to specific neuroprotective interventions. Ongoing investigation into the degree and features of mitochondrial dysfunction after TBI in humans is warranted, and could lead to the development of novel, subcellular, neuroprotective strategies aimed at early and sustained mitochondrial impairment.

MITOCHONDRIA AND THE DEVELOPING BRAIN

Developmental differences in brain mitochondria of normal rats have been well documented. In general, through the first 3–4 weeks of life in the rat, there is a threefold increase in mitochondrial protein per cell, with corresponding increases in respiratory enzyme activity and increasing oxygen consumption (Milstein *et al.*, 1968; Murthy and Rappoport, 1963). There are also potential differences in mitochondrial membrane composition (Sitkiewicz *et al.*, 1982) and relative ratios of synaptosomal to nonsynaptosomal brain mitochondria (Dienel *et al.*, 1977). A series of studies by the laboratory of Holtzman and others have detailed developmental differences in brain mitochondrial activity in immature (<4 weeks old) versus mature (adult) rats. ADP/O ratios with NAD-linked substrates were lower in rats <2 weeks of age, increased between the 3rd and 4th week, and reached adult levels by the 4th week of life (Holtzman and Moore, 1973, 1975).

To understand the role of mitochondrial dysfunction after injury to the developing brain, one must understand developmental aspects of mitochondrial function in normal (uninjured) brain. Initial studies in our lab have compared brain mitochondria isolated from immature rats to those isolated from adult rats. We evaluated Ca^{2+} uptake capacity of isolated mitochondria in both physiologic conditions and in those conditions potentially present after TBI, such as acidosis ($\text{pH} = 6.5$) and ATP depletion. The Ca^{2+} uptake capacity represents resistance to Ca^{2+} -induced mitochondrial injury. In a physiologic environment ($\text{pH} = 7.0$ with ATP), mitochondria isolated from adult rats had a higher Ca^{2+} uptake capacity than mitochondria from immature rats (Fig. 2(A)). Acidosis ($\text{pH} =$

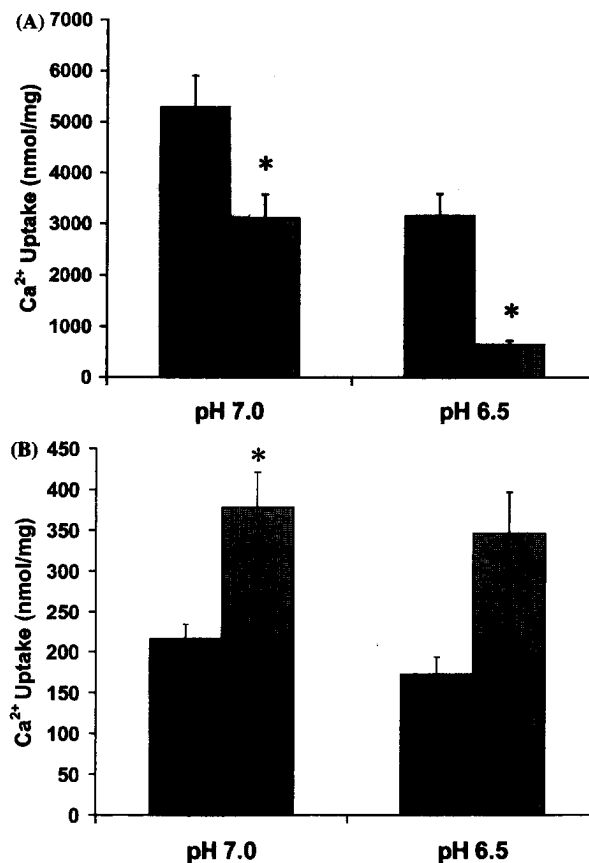


Fig. 2. Calcium uptake capacity of mature and immature brain mitochondria. In the presence of 3 mM ATP (Fig. 2(A)), maximal Ca^{2+} uptake capacity is greater in adult (black bars) versus immature (gray bars) rat brain mitochondria at both pH of 7.0 and 6.5. In the absence of ATP (Fig. 2(B)), maximal Ca^{2+} uptake capacity is greater in immature rat brain mitochondria.

6.5) caused a significant reduction in maximal Ca^{2+} uptake in both immature and adult rat brain mitochondria. As brain tissue acidosis contributes to poor outcome following TBI in both animals and humans, these observations suggest a subcellular mechanism of action that could be particularly important in immature animals and children. In contrast to the differences seen with pH modification, immature rats appear to tolerate the absence of ATP much better than adult rats. At both a pH of 7.0 and 6.5, immature rat brain mitochondria had a greater Ca^{2+} -uptake capacity than adult rat brain mitochondria (Fig. 2(B)). These results suggest that brain mitochondria from immature animals are more resistant than those of mature animals to Ca^{2+} -induced injury under extreme conditions (no ATP) that can occur within some brain cells following TBI.

Animal models of hypoxia-ischemia and traumatic brain injury have shown developmental differences in

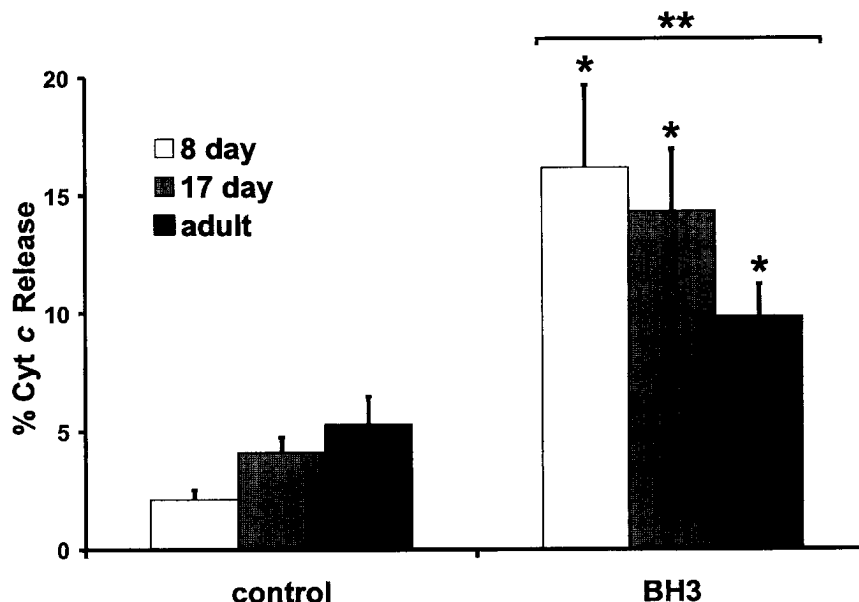


Fig. 3. Release of cytochrome *c* from brain mitochondria isolated from 8-day old, 17-day old, and adult rats in response to BH3 peptide. There is a significant difference between control and BH3 peptide treatment across groups and a significant affect of age on this difference (two-way ANOVA, $p < 0.05$).

apoptotic neuronal death. The exact mechanisms to explain these differences are unknown, but are likely multifaceted and related to mitochondrial response to injury. To begin to evaluate this, we studied the in vitro response of isolated brain mitochondria to proapoptotic peptides (BH3 cell death domain-containing peptide). Analysis by ELISA revealed greater cytochrome *c* release from mitochondria of immature rats exposed to BH3 peptide compared to adult rats, with the youngest rats (8do) showing the greatest release (Fig. 3). Previous studies in other laboratories have suggested that the protein Bax may be required for "BH3 only" proteins to promote cytochrome *c* release (Desagher *et al.*, 1999; Wei *et al.*, 2001), and brain levels may decline during maturation. Using immunoblot analysis, we found significant amounts of detectable Bax in 8do isolated rat forebrain mitochondria and moderate amounts of detectable Bax in 17do rats, but none detectable in adult rat brain mitochondria (Fig. 4).

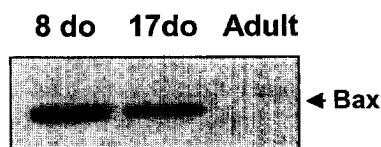


Fig. 4. Immunoblot for Bax in isolated rat brain mitochondria from 8-day-old, 17-day-old, and adult rats.

The presence of endogenous Bax in association with brain mitochondria may represent a potential explanation for the differences observed in sensitivity to BH3-induced cytochrome *c* release. The potentially very important conclusion we have reached from these results is that immature brain mitochondria are "primed" to release cytochrome *c* in response to BH3 domain proteins (e.g., tBid) due to the presence of endogenous mitochondrial Bax (Polster *et al.*, 2003). These characteristics of immature brain mitochondria could help explain the apparently greater contribution of apoptosis to brain cell death following TBI in immature animals.

MITOCHONDRIAL DYSFUNCTION AFTER EXPERIMENTAL TBI IN IMMATURE RATS

Given the important role that mitochondria likely play after TBI, and the unique aspects of mitochondrial development in the immature brain, it stands to reason that mitochondrial dysfunction following TBI in the immature brain would have profound effects. Very few studies have addressed this, although several centers have demonstrated unique patterns after TBI in immature rats, and explanations have discussed mechanisms with importance to mitochondrial function, such as alterations in CBF (Biagas *et al.*, 1996; Grundl *et al.*, 1994) and metabolism

(Thomas *et al.*, 2000). The most comprehensive investigation to date reveals two unique patterns of cell death after TBI in 7d rats, involving excitotoxic and apoptotic mechanisms (Pohl *et al.*, 1999). Neurons adjacent to the site of impact showed changes identical to those induced by glutamate, which peaked at 4 h and was not evident by 24 h. A delayed pattern of apoptotic cell death peaked at 24 h, and accounted for a much greater number of dying cells (2.2 million) than excitotoxicity (16,000). Interestingly, NMDA receptor antagonists protected against the primary excitotoxicity, but increased the severity of secondary apoptotic damage. Administration of SPBN, a free radical scavenger mitigated apoptotic damage. This study clearly demonstrates the importance of independent evaluation of pathologic pathways in the developing brain and supports the potential importance of mitochondrial dysfunction in this unique environment.

MITOCHONDRIA AND NEUROPROTECTION AFTER TBI

With the growing evidence for mitochondrial participation in traumatic neuronal injury, neuroprotective approaches must include strategies aimed to limit and reverse mitochondrial dysfunction. Interventions that have directly targeted mitochondria, such as calcium channel blockade (Verweij *et al.*, 1997, 2000; Xiong *et al.*, 1998) and antioxidant administration (Xiong *et al.*, 1997b, 1998, 1999) have documented reversibility of this mitochondrial dysfunction. Most importantly, animal studies have demonstrated that "mitoprotective" strategies have translated into neuroprotective strategies in models of TBI. Studies by Verweij *et al.* (2000) and Berman *et al.* (2000) initially examined time-window profiles and dose-response curves of the calcium channel blocker Ziconotide after TBI using mitochondrial outcome measures as endpoints. When the optimal mitochondrial dose was administered, rat showed improvements in motor and cognitive testing from 1 to 42 days after TBI. A number of studies involving the mitochondrial PTP inhibitor, cyclosporin A have shown improvement in both mitochondrial function, cerebral metabolism, and tissue damage after TBI (Alessandri *et al.*, 2002; Scheff and Sullivan *et al.*, 1999; Sullivan *et al.*, 1999). These studies suggest an important role for the mitochondrial PTP after TBI, especially with the lack of efficacy of the immunophilin ligand FK506 in one study (Scheff and Sullivan, 1999). However, the calcineurin interaction properties cannot be disregarded, as FK506 does protect against traumatic axonal injury (Singleton *et al.*, 2001). Finally, studies have begun to evaluate the role of uncoupling proteins after brain in-

jury through proposed mechanisms of mild mitochondrial depolarization with resultant reduction in ROS generation. Specifically, overexpression of uncoupling protein-2 has been shown to reduce cortical damage and improve neurologic outcome after TBI in mice (Mattiasson *et al.*, 2003).

CONCLUSION

The importance of mitochondrial dysfunction following TBI in both preclinical and clinical studies is evident. Also evident, from clinical studies, is the extreme heterogeneity of injury following TBI, which can be influenced by age, gender, injury severity, injury mechanism, brain region, and number and degree of secondary insults. From the preclinical studies, cellular and even *sub-cellular* heterogeneity of alterations in metabolism and bioenergetics after TBI has been seen. Development of neuroprotective treatments must take into consideration this variability, and studies must continue to make attempts to understand the molecular mechanisms responsible for neuronal injury in different settings after TBI. This should include rigorous evaluation of important clinical variables, such as patient age, as interventions that are protective in adult models may be ineffective or even detrimental in pediatric TBI (Pohl *et al.*, 1999). Continued study of mitochondrial participation in TBI may ultimately lead to translation into effective neuroprotective interventions targeted at specific patient profiles.

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Mitochondrial Impairment in the Developing Brain after Hypoxia–Ischemia

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The pattern of cell death in the immature brain differs from that seen in the adult CNS. During normal development, more than half of the neurons are removed through apoptosis, and mediators like caspase-3 are highly upregulated. The contribution of apoptotic mechanisms in cell death appears also to be substantial in the developing brain, with a marked activation of downstream caspases and signs of DNA fragmentation. Mitochondria are important regulators of cell death through their role in energy metabolism and calcium homeostasis, and their ability to release apoptogenic proteins and to produce reactive oxygen species. We find that secondary brain injury is preceded by impairment of mitochondrial respiration, signs of membrane permeability transition, intramitochondrial accumulation of calcium, changes in the Bcl-2 family proteins, release of proapoptotic proteins (cytochrome C, apoptosis inducing factor) and downstream activation of caspase-9 and caspase-3 after hypoxia-ischemia. These data support the involvement of mitochondria-related mechanisms in perinatal brain injury.

KEY WORDS: Hypoxia-ischemia; mitochondria; caspases; *N*-methyl-D-aspartate; nitric oxide; apoptosis-inducing factor; cytochrome C; immature; membrane permeability transition; Bcl-2.

INTRODUCTION

Perinatal brain injury subsequent to birth asphyxia remains an important clinical problem. Even though we still lack effective neuroprotective strategies, considerable progress has been made in understanding the pathogenesis of neuronal damage in the immature brain (Johnston *et al.*, 2002; Vannucci *et al.*, 1997).

Thus, it is now well accepted that a cerebral hypoxic–ischemic (HI) event of sufficient severity to deplete tissue energy reserves (primary insult) is often followed by transient but complete restoration of glucose utilization, ATP, and phosphocreatine upon reperfusion/reoxygenation (Blumberg *et al.*, 1997; Gilland *et al.*, 1998a). Thereafter a secondary decrease of high energy phosphates occurs in parallel with a decrease in tissue utilization of glucose, activation of caspase-3, and DNA fragmentation (Blumberg *et al.*, 1997; Wyatt *et al.*, 1989;

Gilland *et al.*, 1998a,b; Puka-Sundvall *et al.*, 2000a). Secondary energy failure develops in most brain regions 6–48 h after the insult in immature animal models.

Several studies indicate that mitochondria play an important role in adult ischemia (Fiskum *et al.*, 1999), but the information about the developing brain is limited. In this brief review, I will describe critical mitochondrial events during the early recovery period and present experimental data in support of the fact that mitochondria may have a critical role in the decision of cellular fate after neonatal HI.

ALTERATIONS OF MITOCHONDRIAL MORPHOLOGY, LOCALIZATION, AND METABOLISM AFTER HI

We recently found that mitochondria (labelled with COX IV) exhibited a “fibrous” pattern of distribution throughout the soma and processes in normal neurons of the cerebral cortex in 7-day-old rats. However, already 2 h after HI, a more punctate or granular appearance of mainly juxta-nuclear COX IV staining was found (Hallin *et al.*,

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unpublished), in agreement with Northington *et al.* (2001). In another study, electron microscopy combined with the oxalate-pyroantimonate technique was used to analyze mitochondrial ultrastructure and intramitochondrial calcium accumulation after HI (Puka-Sundvall *et al.*, 2000b). At 3 h and 30 min after HI, a progressive accumulation of calcium was detected in the endoplasmic reticulum, cytoplasm, nucleus, and, most markedly, in the mitochondrial matrix of neurons. Some mitochondria developed a considerable degree of swelling reaching a diameter of several micrometers at 3 h of reflow, whereas the majority of mitochondria appeared moderately affected. Chromatin condensation was observed in the nuclei of many cells with severely swollen mitochondria with calcium deposits. In conclusion, mitochondrial localization seems to change from a widespread to a more perinuclear distribution after HI, accompanied by mitochondrial swelling and accumulation of calcium in the mitochondrial matrix.

During early recovery after HI high energy phosphates in the cerebral cortex are restored as previously mentioned. During this phase, the 2-deoxyglucose (2-DG) utilization was increased, which correlated with increased levels of tissue lactate (Gilland and Hagberg, 1996) and a depression of mitochondrial respiration (Gilland *et al.*, 1998a). We have also found that post-HI administration of an *N*-Methyl-D-aspartate (NMDA) receptor antagonist normalized 2-DG utilization, lactate levels, improved mitochondrial respiration and attenuated cortical brain injury (Gilland and Hagberg 1996, 1997; Gilland *et al.*, 1998a,b). These data suggest that NMDA-receptor activation in the early recovery phase depresses mitochondrial respiration with a compensatory increase of anaerobic glucose cycling to lactate, which precedes development of cortical brain injury. Interestingly, a similar pattern of increased glucose use occurred in the CNS of asphyxiated infants, particularly in brain regions that were subsequently injured (Blennow *et al.*, 1995). Such an increase in glucose utilization occurred in parallel with marked elevations of glutamate in the cerebrospinal fluid (Hagberg *et al.*, 1993), implying that HI brain injury also in postasphyxiated infants is preceded by a phase of mitochondrial impairment related to activation of excitatory amino acid receptors.

MEMBRANE PERMEABILITY TRANSITION (MPT)

In isolated mitochondria, MPT is associated with a nonspecific permeabilization of the inner mitochondrial membrane, resulting in a dramatic swelling of the mitochondria, followed by rupture of the outer membrane

(Ravagnan *et al.*, 2002). Experimental studies have found evidence for MPT after ischemia in the adult brain, and MPT blockers have been shown to be potent neuroprotectants (Friberg and Wieloch, 2002). In immature rats, the above-mentioned ultrastructural changes are compatible with MPT (Puka-Sundvall *et al.*, 2001). To investigate this further, [¹⁴C]2-deoxyglucose (DOG) was administered to control animals, at various time points after HI, and MPT was measured as entrapment of DOG-6-P in mitochondria (Griffiths and Halestrap, 1995). A significant increase in DOG-6-P in mitochondria indicated that MPT occurred in two phases: a primary MPT after 0–1.5 h and a secondary MPT after 6.5–8 h of reperfusion (Puka-Sundvall *et al.*, 2001). We also found a loss of mitochondrial glutathione during early and late recovery (Wallin *et al.*, 2000), offering additional support of a biphasic increase of mitochondrial permeability after HI. However, in contrast to the adult, the MPT blocker cyclosporine A did not affect brain injury or mitochondrial respiration in the neonatal brain.

Intrinsic Apoptotic Pathway and Caspase Activation

Studies performed on cultured cells, cell-free systems, or purified mitochondria suggest that mitochondria regulate apoptotic cell death through their capacity to release proapoptotic proteins (Ravagnan *et al.*, 2002). Cytochrome C, and other apoptogenic proteins, such as apoptosis-inducing factor (AIF), endonuclease G, SMAC/Diablo, and HtrA2/Omi, can be released from the mitochondrial intermembrane space (Ravagnan *et al.*, 2002). Data suggest that Bax, Bad, Bid, and other members of the Bcl-2 family are involved in the regulation of mitochondrial release of proapoptotic proteins. Cytochrome C interacts with APAF-1, ADP, and procaspase-9 to form the heptameric apoptosome, leading to activation of caspase-9, which in turn cleaves and activates pro-caspase-3. AIF, on the other hand, promotes apoptosis in a caspase-independent manner (Susin *et al.*, 1999).

Many of the key elements of apoptosis have been demonstrated to be strongly upregulated in the immature brain, such as caspase-3 (Blomgren *et al.*, 2001), APAF-1 (Ota *et al.*, 2002), Bcl-2 (Merry *et al.*, 1994), and Bax (Vekrellis *et al.*, 1997). Caspase-3 is markedly activated after HI in the immature brain (Cheng *et al.*, 1998; Wang *et al.*, 2001; Zhu *et al.*, 2000) and cells with the cleaved active form of caspase-3 colocalize with markers of DNA fragmentation in injured brain regions (Zhu *et al.*, 2000). Caspase-3 inhibitors (Cheng *et al.*, 1998) as well as transgenic overexpression of X-linked inhibitor of apoptosis (XIAP) (Wang *et al.*, 2004) attenuate

caspase-3 activation and provide a considerable degree of neuroprotection in the neonatal setting.

It is not known if the extrinsic or intrinsic (mitochondrial) pathway is responsible for the downstream activation of caspase-3. However, assembly of the apoptosome is easily induced in homogenates from the immature (but not adult) brain (Gill *et al.*, 2002), cytochrome C is released to the cytosol in response to HI (Northington *et al.*, 2001; Zhu *et al.*, 2003) and caspase-9 is activated (Northington *et al.*, unpublished; Hallin *et al.*, unpublished). In addition, other proapoptotic proteins like AIF (Zhu *et al.*, 2003), SMAC/Daiblo (Wang *et al.*, 2004), and HtrA2/Omi (Wang *et al.*, 2004) translocate from the mitochondria to a nuclear localization, suggesting that proapoptotic proteins are indeed released during the early recovery phase after HI. We find that cells with immunohistochemical translocation of cytochrome C and AIF often exhibit signs of DNA fragmentation (detected with a hairpin probe) and nuclear condensation, and these cells are preferentially localized in regions with early loss of the neuronal marker MAP-2 (Zhu *et al.*, 2003). Smac and HtrA2 translocation also occurred predominantly in injured areas, and immunostaining often occurred in cells with nuclear condensation or pyknosis (Wang *et al.*, 2004). These data show an association between mitochondrial release of proapoptotic proteins and brain injury, but their direct role in the process leading to cell death remains to be clarified.

BCL-2 FAMILY OF PROTEINS AND NEONATAL HI

There is also evidence for involvement of the Bcl-2 family of proteins. Transgenic mice overexpressing human Bcl-xL postnatally were dramatically resistant to neonatal HI- and axotomy-induced apoptosis (Parsadanian *et al.*, 1998). In addition, HI induced an increase in Bax in mitochondrial-enriched cell fractions, which occurred in parallel with an increase of cytochrome C in the cytosol preceding activation of caspase-3 in the neonatal thalamus (Northington *et al.*, 2001). Furthermore, HI brain injury seems to be attenuated in Bax gene-deficient mice compared to wild-type controls (Gibson *et al.*, 2001). We recently found that Bax translocation to mitochondria after HI, was accompanied by an increased nuclear staining of Bcl-2 (Hallin *et al.*, unpublished). Using a site-specific antibody for phosphorylation of Bcl-2 at serine-24 (PS24-Bcl-2), it was found that the number of cells positive for PS24-Bcl-2 increased during 3–24 h of reperfusion in all investigated brain areas after neonatal HI. Phosphorylation of Bcl-2 coincided with cytochrome C translocation and

colocalized with, but preceded, caspase-3 activation. In summary, Bcl-2 is phosphorylated (inactivated?) and translocated to the nucleus, concomitant with increased mitochondrial Bax immunoreactivity, cytochrome C release, and activation of caspase-3. Furthermore, ceramide “preconditioning-like” protection in the neonatal setting was accompanied by upregulation of both Bcl-2 and Bcl-xL (Chen *et al.*, 2001), offering additional support for involvement of Bcl-2 family proteins and mitochondria in the determination of susceptibility of the immature brain.

PARP-1 AND AIF

PARP-1 is a DNA repair enzyme that has been demonstrated to be critically involved in ischemic brain injury in the adult (Yu *et al.*, 2003). Mice with PARP-1 gene disruption are resistant to ischemia (Eliasson *et al.*, 1997), and PARP inhibitors provide protection (Ducrocq *et al.*, 2000; Yu *et al.*, 2003). We recently found that PARP-1 gene deficiency also confers protection in neonatal mice (Hagberg *et al.*, 2004). PARP-1-mediated cell death has previously been explained in terms of NAD⁺ consumption and mitochondrial energy failure (Eliasson *et al.*, 1998). A recent study suggests that PARP-1 mediates the release of AIF from mitochondria, resulting in caspase-independent cell death, a process which could be blocked by microinjection of an antibody against AIF (Yu *et al.*, 2002). In support of this hypothesis, we found that cells with increased PAR immunoreactivity after HI (indicative of activation of PARP-1) often exhibit a shift in AIF immunoreactivity from the mitochondria to the nucleus. Irrespective of which molecular mechanisms prove to be most important, it seems likely that mitochondria are important in PARP-1-mediated cell death (Yu *et al.*, 2002, 2003).

NMDA-RECEPTOR ACTIVATION, NITRIC OXIDE (NO), AND MITOCHONDRIAL IMPAIRMENT

There are also other potential adverse factors in the mitochondrial environment that could be important (Fig. 1). NMDA receptor activation with increased Ca²⁺ influx, free radical formation, and induction of NO could damage mitochondrial membranes (Crow and Beckman, 1995; Nowicki *et al.*, 1982; Schulz *et al.*, 1995; Zaidan and Sims, 1994). As previously mentioned, administration of NMDA receptor antagonists prevents the depression mitochondrial respiration *in vivo* (Gilland and Hagberg, 1996). In addition, we have found that both NMDA receptor blockers (Puka-Sundvall, 2000c) and a combined inhibitor of inducible and neuronal NO

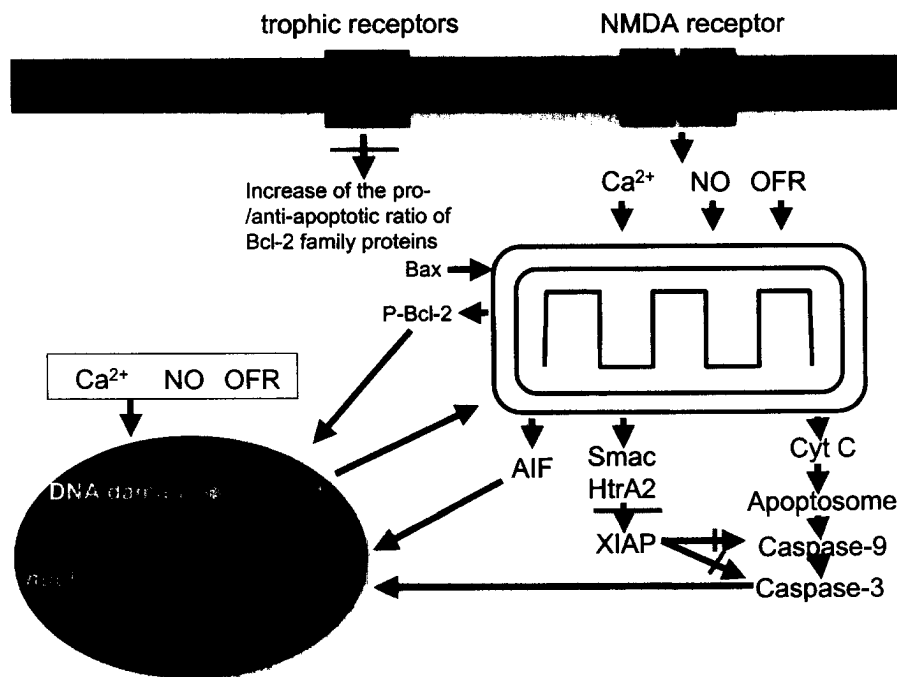


Fig. 1. Tentative role of mitochondria in cellular injury after HI in the developing CNS. AIF, Apoptosis-inducing factor; Bax, Bcl-2-associated protein X; Bcl-2, B-cell leukemia gene 2; CytC, cytochrome C; HtrA2, high-temperature requirement serine protease A2; OFR, oxygen free radicals; Smac, second mitochondrial activator of caspase; XIAP, X-linked inhibitor of apoptosis.

synthase (2-iminobiotin) (Peeters-Scholte, 2002) inhibit activation of caspase-3, DNA fragmentation, and brain injury (Gilland and Hagberg, 1997). These data suggest indirectly that glutamate activation of NMDA receptors and production of NO may contribute to the mitochondrial release of proapoptotic proteins and subsequent activation of caspase-3.

CONCLUSION

Neonatal HI induces activation of NMDA receptors, production of NO/oxygen free radicals, and loss of trophic factor support, speculatively leading to an increase in the ratio of proapoptotic/antiapoptotic Bcl-2 family proteins. The changed environment will lead to depression of mitochondrial respiration, intramitochondrial Ca^{2+} accumulation and swelling, mitochondrial permeability transition, and release of proapoptotic proteins, resulting in caspase-dependent and caspase-nondependent cell death (Fig. 1).

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MPTP as a Mitochondrial Neurotoxic Model of Parkinson's Disease

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent neurotoxin extensively used to model Parkinson's disease (PD). A cascade of deleterious events, in which mitochondria play a pivotal role, drives MPTP neurotoxicity. How mitochondria are affected by MPTP and how their defect contributes to the demise of dopaminergic neurons in this model of PD are discussed in this review.

KEY WORDS: MPTP; Parkinson's disease; neurodegeneration; mitochondria; oxidative stress; ATP depletion; programmed cell death.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects (Ziering *et al.*, 1947). MPTP can induce a parkinsonian syndrome in humans and nonhuman primates almost indistinguishable from Parkinson's disease (PD) on both clinical and neuropathological standpoints (Langston and Irwin, 1986). Over the years, MPTP has been used in a host of different animal species, especially in mice (Heikkilä *et al.*, 1989), to recapitulate the hallmark of PD cellular pathology, namely the degeneration of the nigrostriatal dopaminergic pathway (Dauer and Przedborski, 2003). Although the MPTP model departs from PD on several significant aspects, it continues to be regarded as the best experimental model of this common neurodegenerative disease. With respect to PD, enthusiasm for the MPTP model is driven by the belief that unraveling the MPTP neurotoxic process in animals may provide hints into the mechanisms responsible for the demise of dopaminergic neurons in human PD.

Various key cellular and molecular components underlying the MPTP neurotoxic process have been re-

viewed in details in the following references (Dauer and Przedborski, 2003; Przedborski and Vila, 2003) and will thus not be discussed here. Instead, the focus of this minireview will be devoted to the role of the mitochondria in the deleterious effects of the parkinsonian toxin MPTP.

FIRST STEP FIRST

MPTP is a protoxin whose toxicokinetics is a complex, multistep process (Dauer and Przedborski, 2003). As indicated by its octanol/water partition coefficient of 15.6 (Riachi *et al.*, 1989), MPTP is a highly lipophilic molecule, which is able to readily permeate lipid bilayer membranes. It is therefore not surprising to observe that MPTP crosses the blood-brain barrier in a matter of seconds after its systemic administration (Markey *et al.*, 1984). Once in the brain, it is rapidly converted into 1-methyl-4-phenylpyridinium (MPP⁺), the actual neurotoxin (Heikkilä *et al.*, 1984). This critical transformation of MPTP into MPP⁺ is a two-step process. First, MPTP undergoes a two-electron oxidation, catalyzed by monoamine oxidase B (MAO-B), yielding the intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) (Chiba *et al.*, 1984). Given the discrete cellular distribution of MAO-B in the brain (Kitahama *et al.*, 1991), it is believed that the conversion of MPTP to MPDP⁺ occurs specifically in glial and serotonergic cells, and not in dopaminergic neurons. MPDP⁺ is an unstable molecule which readily undergoes spontaneous

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disproportionation to MPP^+ and MPTP (Chiba *et al.*, 1985; Peterson *et al.*, 1985).

Once formed, MPP^+ is presumably released from glial and serotonergic cells into the extracellular space prior to entering dopaminergic neurons. Yet, MPP^+ has an octanol/water partition coefficient of 0.09 (Riachi *et al.*, 1989), which indicates that, while being a lipophilic cation, MPP^+ is far less lipophilic than MPTP. Thus, unlike MPTP, MPP^+ is most likely unable to easily diffuse across cellular lipid bilayer membranes. Instead, it is to be expected that the release of MPP^+ from its intracellular sites of formation and entry into adjacent neurons depend on specialized carriers. Consistent with this view is the fact that MPP^+ access to dopaminergic neurons relies on the plasma membrane dopamine transporter (Bezard *et al.*, 1999; Javitch *et al.*, 1985).

MITOCHONDRIAL ACCUMULATION

Once inside neurons, MPP^+ rapidly accumulates in the mitochondrial matrix (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). Initially, it was thought that MPP^+ gains access to the mitochondrial matrix through a carrier (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). However, it is now well established that MPP^+ is passively transported (Davey *et al.*, 1992; Hoppel *et al.*, 1987) by a mechanism relying entirely upon the large mitochondrial transmembrane potential gradient ($\Delta\psi$) of -150 to -170 mV (Aiuchi *et al.*, 1988; Davey *et al.*, 1992; Hoppel *et al.*, 1987; Ramsay *et al.*, 1986; Ramsay and Singer, 1986).

Like with other lipophilic cations (Rottenberg, 1984), the higher the concentrations of intramitochondrial MPP^+ , the lower the $\Delta\psi$ and, consequently, the slower the uptake of extramitochondrial MPP^+ (Davey *et al.*, 1992; Hoppel *et al.*, 1987). The demonstration that the ion-pairing agent tetraphenylboron anion increases both the rate and the extent of MPP^+ uptake in isolated mitochondria (Aiuchi *et al.*, 1988; Davey *et al.*, 1992; Hoppel *et al.*, 1987) further supports this concept. As discussed below, MPP^+ inhibits mitochondrial respiration, which likely also contributes to the loss of the $\Delta\psi$ gradient and to the dampening of the mitochondrial uptake of MPP^+ . It is thus not surprising that the accumulation of MPP^+ by energized mitochondria behaves as a saturable phenomenon in the presence of high extramitochondrial concentrations of MPP^+ (e.g., >10 mM) (Ramsay and Singer, 1986) and appears to reach a steady state after a few minutes (Davey *et al.*, 1992; Ramsay *et al.*, 1986). This apparent steady state persists until mitochondrial suspension becomes anaerobic or $\Delta\psi$ is collapsed by the addition of an uncoupler agent such

as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Ramsay *et al.*, 1986). Remarkably, energized mitochondria incubated with 0.5 mM MPP^+ reach matrix concentrations of more than 24 mM after only 10 min (Ramsay and Singer, 1986). This fast and avid uptake suggests that most, if not all, of the cytosolic MPP^+ would eventually accumulate in the mitochondrial matrix after the systemic injection of MPTP.

INTRAMITOCHONDRIAL MPP^+

It is well established that intramitochondrial MPP^+ inhibits oxidative phosphorylation (Nicklas *et al.*, 1985; Singer *et al.*, 1987). Intramitochondrial MPP^+ also appears to inhibit the tricarboxylic acid cycle enzyme α -ketoglutarate dehydrogenase (Mizuno *et al.*, 1987a). Although both mitochondrial metabolic alterations may contribute to MPP^+ cytotoxicity, attention has been paid almost exclusively to the action of MPP^+ on the respiratory chain.

It is well documented that MPP^+ impairs, in a dose- and time-dependent manner, the ADP-stimulated oxygen consumption (State 3) in intact mitochondria supported by the NADH-linked substrates glutamate and malate (Mizuno *et al.*, 1987b; Nicklas *et al.*, 1985). MPP^+ is, however, ineffective in inhibiting the oxygen consumption in mitochondria supported by succinate (Mizuno *et al.*, 1987b; Nicklas *et al.*, 1985). Furthermore, MPP^+ prevents the binding of the classical Complex I inhibitor [^{14}C]-rotenone to electron transport particles (Ramsay *et al.*, 1991a). Collectively these findings indicate that MPP^+ , like rotenone and piericidin A, impairs mitochondrial respiration by inhibiting the multi-subunit enzyme Complex I (i.e., NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain. This straightforward interpretation is supported by the electron spin resonance demonstration that MPP^+ does actually bind to Complex I and blocks the terminal step of electron transfer from the highest potential iron-sulfur cluster of Complex I called N2 to ubiquinone (Ramsay *et al.*, 1987).

The use of several MPP^+ analogs and cationic inhibitors has demonstrated that MPP^+ binds at two distinct sites within the mitochondrial electron transport chain region comprised between N2 and ubiquinone (Gluck *et al.*, 1994; Miyoshi *et al.*, 1997, 1998; Ramsay *et al.*, 1989, 1991b; Ramsay and Singer, 1992). These studies have also demonstrated that the occupation of both sites appears to be required for complete inhibition of NADH oxidation. The binding of MPP^+ to the first, more *hydrophilic* site seems to primarily affect the functional coupling between the PSST and the ND1 subunit of Complex I and to account

for only 40% of the MPP^+ -induced reduction in NADH oxidation (Schuler and Casida, 2001). The binding of MPP^+ to the second, more *hydrophobic* site seems quite potent in blocking Complex I enzymatic activity (Schuler and Casida, 2001). Yet, the exact location of this second binding site in Complex I remains to be determined. Nonetheless, the importance of the binding to PSST, but not to the ND1 subunit in the inhibition of Complex I-mediated NADH oxidation (Schuler *et al.*, 1999; Schule and Casida, 2001), suggests that the MPP^+ *hydrophobic* site must also be situated somewhere in the PSST subunit. This *hydrophobic* site appears not to exist for other typical Complex I inhibitors such as rotenone and piericidin A (Schuler and Casida, 2001). Accordingly, while MPP^+ binds to Complex I, as do rotenone and piericidin A (Gluck *et al.*, 1994; Krueger *et al.*, 1993), it may not bind to exactly the same Complex I subunit or subunit part as these two other Complex I inhibitors. Also worth noting is the fact that MPP^+ , compared to rotenone and piericidin A, is a far weaker inhibitor of Complex I, which may explain why millimolar concentrations of MPP^+ are needed to inhibit NADH-oxidation in electron transport particles (Hoppel *et al.*, 1987).

CONSEQUENCES OF MPP^+ -INDUCED COMPLEX I INHIBITION

In response to MPP^+ binding to Complex I, the flow of electrons along the respiratory chain is hampered in both dose- and time-dependent manners (Hasegawa *et al.*, 1990; Nicklas *et al.*, 1985; Vyas *et al.*, 1986). The importance of the inhibition of Complex I in the MPTP-induced neurotoxicity *in vivo* is supported by the demonstration that strategies aimed at stimulating oxidative phosphorylation via by-passing the blockade of Complex I not only improve mitochondrial respiration but also mitigate dopaminergic neurodegeneration in mice (Tieu *et al.*, 2003).

The current hypothesis on MPTP cytotoxicity posits that one of the main contributors to cell death is the impaired synthesis of ATP resulting from the inhibition of Complex I by MPP^+ . Relevant to this view is the fact that MPP^+ indeed causes a rapid and profound depletion of cellular ATP levels in isolated hepatocytes (Di Monte *et al.*, 1986), in brain synaptosomal preparations (Scotcher *et al.*, 1990), and in whole mouse brain tissues (Chan *et al.*, 1991). It appears, however, that Complex I activity should be reduced by more than 50% to cause significant ATP depletion in nonsynaptic brain mitochondria (Davey and Clark, 1996). Furthermore, *in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain

ATP levels (Chan *et al.*, 1991). These facts argue against MPP^+ -related ATP deficits being the sole factor underlying MPTP-induced cell death.

Another consequence of Complex I inhibition by MPP^+ is an increased production of reactive oxygen species (ROS). It was shown that incubation of MPTP with brain mitochondria resulted in an oxygen-dependent formation of ROS (Rossetti *et al.*, 1988). It was also shown that incubation of MPP^+ with bovine heart submitochondrial particles causes a production of superoxide radicals when MPP^+ is used at the concentrations expected to be found inside neurons after MPTP systemic administration (Hasegawa *et al.*, 1990). In this study, the authors also demonstrate that the degree of Complex I inhibition is proportional to the amount of superoxide radical produced (Hasegawa *et al.*, 1990). Because modulations of key mitochondrial ROS scavengers, such as manganese superoxide dismutase, affect MPTP-induced neurotoxicity in mice (Andreassen *et al.*, 2001; Klivenyi *et al.*, 1998), it is reasonable to assert that MPP^+ -related ROS production also contributes to MPTP-induced cell death.

CONCLUSION

As discussed above, ATP depletion and ROS overproduction appear to occur soon after MPTP injection, subjecting the intoxicated cells, early on, to an energy crisis and oxidative stress. However, the time course of these perturbations reviewed in the following reference (Przedborski and Vila, 2003) appears to correlate poorly with the time course of neuronal death *in vivo* (Jackson-Lewis *et al.*, 1995). What this meta-analysis is suggesting is that only a few neurons are probably succumbing to the early combined effects of ATP depletion and ROS overproduction. Instead, mounting evidence discussed in the following references (Dauer and Przedborski, 2003; Przedborski and Vila, 2003) indicates that rather than killing the cells, alterations in ATP synthesis and ROS production are pivotal in triggering cell-death-related molecular pathways which, once activated, rapidly lead to the demise of the intoxicated neurons.

Interestingly enough, among these latter molecular pathways, it appears that the mitochondrial-dependent programmed cell death machinery plays a critical role (Vila *et al.*, 2001). As illustrated in Fig. 1, it is thus plausible that the death of neurons caused by MPTP results from a circular cascade of deleterious events starting at the mitochondria by the alteration of the oxidative phosphorylation and finishing also at the mitochondria by the activation of the programmed cell death machinery. Whether the whole circuit depicted above is entirely orchestrated

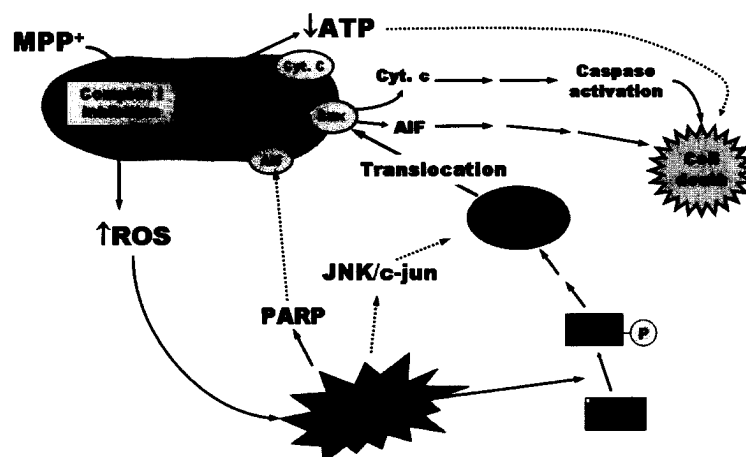


Fig. 1. Illustration of the proposed circular nature of the MPP^+ -mediated cell death cascade. MPP^+ enters in the mitochondrion and binds to Complex I, whereby it inhibits ATP synthesis and stimulates ROS production. These two initial events lead to a host of cellular perturbations such as DNA damage, which, in turn, trigger a variety of cell-death-related pathways. These include activations of p53 by phosphorylation (p53-p) and JNK/c-Jun, which lead to Bax induction and translocation to the mitochondria. DNA damage also stimulates poly(ADP-ribose) polymerase (PARP) activity. Bax translocation and PARP activation promote the translocation of cytochrome *c* and apoptosis-inducing factor (AIF) from the mitochondria to the cytosol. Once in the cytosol, cytochrome *c* participates in a caspase-dependent cell death process, while AIF participates in a caspase-independent cell death process, both of which are not necessary mutually exclusive. Solid arrow, known mechanism; dashed arrow, speculated mechanism.

at the level of the mitochondria or whether it also involves perturbations that arise in the cytosol (e.g., protein nitration, cyclooxygenase-2 induction) and the nucleus (e.g., DNA damage, PARP activation) of the intoxicated cells is the focus of several ongoing studies in our laboratory.

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Mitochondrial Dysfunction and Oxidative Damage in Alzheimer's and Parkinson's Diseases and Coenzyme Q₁₀ as a Potential Treatment

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There is substantial evidence that mitochondrial dysfunction and oxidative damage may play a key role in the pathogenesis of neurodegenerative disease. Evidence supporting this in both Alzheimer's and Parkinson's diseases is continuing to accumulate. This review discusses the increasing evidence for a role of both mitochondrial dysfunction and oxidative damage in contributing to β -amyloid deposition in Alzheimer's disease. I also discuss the increasing evidence that Parkinson's disease is associated with abnormalities in the electron transport gene as well as oxidative damage. Lastly, I reviewed the potential efficacy of coenzyme Q as well as a number of other antioxidants in the treatment of both Parkinson's and Alzheimer's diseases.

KEY WORDS: Mitochondria; oxidative damage; free radicals; Alzheimer's; Parkinson's; coenzyme Q₁₀.

ALZHEIMER'S DISEASE TRANSGENIC MODELS: β -AMYLOID, ENERGY METABOLISM AND TRANSGENIC MODELS

There is a large body of evidence implicating impaired energy metabolism and oxidative damage in the pathogenesis of AD. α -Ketoglutarate dehydrogenase complex activity is severely decreased in postmortem AD brain (Gibson *et al.*, 1998). This is unlikely simply to be secondary to cell loss, because the defect is also found in skin fibroblasts (Sheu *et al.*, 1994). It however could be related vulnerability of the enzyme to oxidative damage. Genetic polymorphisms in one of the key components of α -ketoglutarate dehydrogenase dihydrolipoamide dehydrogenase is associated with AD (Kanamori *et al.*, 2003). A truncated gene product was recently found, which is localized to the intermembrane space of mitochondria. If expression of the truncated gene product is reduced, there is a marked decrease in amounts of subunits of complexes I and IV of the mitochondrial electron transport chain and

a decline of activity (Kanamori *et al.*, 2003). There is a link between mitochondrial abnormalities and oxidative stress in AD postmortem tissue (Hirai *et al.*, 2001), and oxidative damage occurs early in the pathogenesis of AD (Nunomura *et al.*, 2001). Oxidative damage to lipids precedes β -amyloid deposition in a transgenic mouse model of AD (Pratico *et al.*, 2001).

There is also a large body of evidence implicating β -amyloid in the pathogenesis of AD. All genes thus far identified as causing AD are involved with the processing of β -amyloid. Trisomy 21 inevitably results in AD pathology (Olson and Shaw, 1969), and the amyloid precursor protein (APP) gene is located on chromosome 21 (Kang *et al.*, 1987). Mutations in the APP gene result in early onset autosomal dominant AD (Chartier-Harlin *et al.*, 1991; Mullan *et al.*, 1992). Mutations in presenilins, which also cause early onset autosomal dominant AD (Wisniewski *et al.*, 1997), increase levels of the particularly fibrillogenic species $A\beta_{42}$ (Borchelt *et al.*, 1996; Duff *et al.*, 1996), through an effect on the γ -secretase (Strooper *et al.*, 1998; Wolfe *et al.*, 1999). Finally, the $\epsilon 4$ allele of apolipoprotein E (apoE) increases the risk of late onset AD (Strittmatter *et al.*, 1993), and apoE4 binds directly to $A\beta$ and promotes its fibrillogenesis (Castano *et al.*, 1995).

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There are strong links between the mitochondrial and amyloid hypotheses. On one hand, mitochondrial dysfunction and oxidative stress may alter APP processing, leading to increased intracellular A β accumulation. Inhibition of cytochrome oxidase results in accumulation of potentially amyloidogenic C-terminal fragments (Gabuzda *et al.*, 1994). Free radical stress increases cellular A β_{42} levels (Ohya *et al.*, 2000). Uncoupling mitochondria with FCCP in normal astrocytes recapitulates the altered APP processing and intracellular accumulation of A β_{42} seen in astrocytes and neuronal cultures from fetal Down's syndrome brain (Busciglio *et al.*, 2002). There is also evidence that oxidative stress increases the activity of β -secretase, the enzyme responsible for N-terminal cleavage of β -amyloid from the amyloid precursors protein (Drake *et al.*, 2003; Tamagno *et al.*, 2002). In Down's syndrome, there is evidence that oxidative damage precedes β -amyloid deposition (Nunomura *et al.*, 2000).

On the other hand, β -amyloid may cause mitochondrial dysfunction and oxidative stress. β -Amyloid suppress mitochondrial succinate dehydrogenase and inhibits of PC12 cell redox activity (Kaneko *et al.*, 1995; Sherman *et al.*, 1994). Exposure of isolated rat brain mitochondria to β -amyloid caused a significant reduction in state 3 and state 4 respiration (Casley *et al.*, 2002). β -Amyloid protein induces oxidative damage to mitochondrial DNA in PC12 cells (Bozner *et al.*, 1997), and there is increased generation of reactive oxygen species in neurons cultured from fetal Down's syndrome (Busciglio and Yankner, 1995). Recently, a direct link between the mitochondrial and amyloid hypotheses was demonstrated, by showing that APP is physically targeted to mitochondria and impairs mitochondrial function in neuronal cells (Anandatheerthavarada *et al.*, 2003).

There are now several transgenic animal models which show increased β -amyloid deposition. These include transgenic mice overexpressing APP with the V717F mutation, and the Swedish double mutation at positions 670/671 (Tg2576) (Hsiao *et al.*, 1996; Masliah *et al.*, 1996; Sturchler-Pierrat *et al.*, 1997). These mice are analogous to recently described mice. These TgCRND8 mice have a double mutant form of the amyloid precursor protein 695 (KM 670/671 NL and V717F), under control of the PrP gene promoter (Chishti *et al.*, 2001). The mice show thioflavin S-positive β -amyloid deposits at 3 months of age, and dense-cored plaques and neuritic pathology from 5 months of age. In the Tg2576 mice, β -amyloid deposits are associated with evidence of oxidative stress as assessed by immunostaining (Pappolla *et al.*, 1998; Smith *et al.*, 1998), and oxidative damage to lipids appears to precede β -amyloid deposition in AD transgenic mice (Pratico *et al.*, 2001).

A recent paper showed that intracellular accumulated β -amyloid precedes both neurofibrillary tangles and synaptic dysfunction in a transgenic mouse expressing β -amyloid, presenilin, and tau mutations (Oddo *et al.*, 2003). We examined the effects of crossing mice with a partial deficiency of manganese superoxide dismutase with Tg1995 mice (William *et al.*, 1998). This markedly exacerbated β -amyloid deposition, providing direct evidence of a link between β -amyloid deposition and oxidative damage.

MITOCHONDRIAL DYSFUNCTION IN PD

The possible role of oxidative damage and mitochondrial dysfunction in PD has been strengthened by the finding that chronic infusions of the complex I inhibitor rotenone produce an animal model of PD in rats (Betarbet *et al.*, 2000). The infusions produced a selective loss of substantia nigra dopaminergic neurons as well as cytoplasmic α -synuclein immunoreactive inclusions closely resembling Lewy bodies. The mechanisms of neurotoxicity appears to involve oxidative damage (Scherer *et al.*, 2002).

Evidence for mitochondrial dysfunction of idiopathic PD comes from a 30–40% decrease in complex I activity in the substantia nigra (Bindoff *et al.*, 1989; Janetzky *et al.*, 1994; Mann *et al.*, 1992; Schapira *et al.*, 1990). Reduced staining for complex I subunits in PD substantia nigra, but preserved staining for subunits of the other electron transport complexes, has been demonstrated immunohistochemically (Hattori *et al.*, 1991). Strong support for a mitochondrial DNA encoded defect comes from studies which showed that complex I defects from PD platelets are transferable into mitochondrial deficient cell lines (Gu *et al.*, 1998; Swerdlow *et al.*, 1996). These defects are associated with increased free radical production, increased susceptibility to MPP⁺, and impaired mitochondrial calcium buffering (Sheehan *et al.*, 1997). Direct sequencing of mitochondrial complex I and tRNA genes failed to show homoplasmic mutations (Simon *et al.*, 2000).

A number of other recent studies, however, provide genetic evidence that mitochondrial DNA abnormalities may contribute to PD pathogenesis. An out-of-frame cytochrome *b* gene deletion occurred in a patient with parkinsonism was associated with increased free radical production (Rana *et al.*, 2000). A novel mitochondrial 12 SrRNA point mutation was found in a pedigree with parkinsonism, deafness, and neuropathy (Thyagarajan *et al.*, 2000). We found parkinsonism occurred in association with the Leber's optic atrophy mitochondrial mutation G11778A (Simon *et al.*, 1999). An increase in mitochondrial DNA deletions/rearrangements and novel complex I mutations were found in the substantia nigra of PD patients

(Gu *et al.*, 2000; Richter *et al.*, 2002). Lastly mitochondrial haplotypes in Caucasian patients (classified as haplotype J) markedly reduce the risk of developing PD (Van der Walt *et al.*, 2003). The mRNA for the NDI subunit of mitochondrial complex I is reduced by 25% in the substantia nigra melanized neurons in PD (Kingsbury *et al.*, 2001).

OXIDATIVE DAMAGE IN PD

A great deal of interest has focused on the possibility that oxidative damage may play a role in the pathogenesis of PD. There are studies showing increased levels of malondialdehyde and cholesterol lipid hydroperoxides, markers for lipid peroxidation, in PD substantia nigra (Dexter *et al.*, 1989, 1994). There are widespread increases in protein carbonyls in PD postmortem brain tissue (Alam *et al.*, 1997). Concentrations of 8-hydroxy-2-deoxyguanosine, a marker of oxidative damage to DNA, are significantly increased in PD substantia nigra and striatum (Alam *et al.*, 1997; Sanchez-Ramos *et al.*, 1994; Zhang *et al.*, 1999). There is evidence for nitrosyl radicals in PD substantia nigra (Shergill *et al.*, 1996). Another means of looking for oxidative stress is to measure concentrations of reduced glutathione. Reduced glutathione is decreased in PD substantia nigra by approximately 50% (Perry *et al.*, 1982; Perry and Yong, 1986; Riederer *et al.*, 1989; Soficm *et al.*, 1992). Individuals with incidental Lewy body disease may have presymptomatic PD, and they have a 35% reduction in reduced glutathione as compared with age-matched controls (Dexter *et al.*, 1994).

Other studies showed an increase in oxidative damage to cytoplasmic DNA and RNA in substantia nigra in PD as detected using immunocytochemistry (Zhang *et al.*, 1999). An increase in oxidative damage to DNA was also reported in leukocytes, serum, and CSF of PD patients (Kikuchi *et al.*, 2002; Migliore *et al.*, 2002). An increase in 3-nitrotyrosine immunoreactivity was reported in Lewy bodies in PD (Good *et al.*, 1998). This finding was confirmed with antibodies specific for nitrated α -synuclein (Giasson *et al.*, 2000). This finding provides a link between oxidative damage and protein aggregates, which are characteristic features of PD. Strengthening this is the observation that intracellular production of peroxynitrite induces α -synuclein aggregation (Paxinou *et al.*, 2001). Other evidence shows that oxidative damage impairs ubiquitination and degradation of proteins by the proteasome (Jenner, 2003).

COENZYME Q₁₀ AND NEUROPROTECTION

There is increasing interest in the potential usefulness of coenzyme Q₁₀ (CoQ₁₀) to treat neurodegenerative

diseases. CoQ₁₀ serves as an important cofactor of the electron transport chain, where it accepts electrons from complexes I and II (Bayer, 1992; Dallner and Sindelar, 2000). CoQ₁₀, which is also known as ubiquinone, serves as an important antioxidant in both mitochondria and lipid membranes. It mediates some of its antioxidant effects through interactions with α -tocopherol (Bayer, 1992; Noack *et al.*, 1994). Coenzyme Q₁₀ blocks apoptosis by inhibiting activation of the mitochondrial permeability transition independently of its free radical scavenging activity (Papucci *et al.*, 2003). Another potential neuroprotective mechanism of coenzyme Q₁₀ is as a cofactor of mitochondrial uncoupling proteins (Echtay *et al.*, 2000, 2002). Coenzyme Q₁₀ is also an obligatory cofactor for mitochondrial uncoupling proteins (Echtay *et al.*, 2000, 2002). Activation of these proteins reduces mitochondrial-free radical generation. Coenzyme Q induces mitochondrial uncoupling in the substantia nigra of primates, and this is associated with marked neuroprotection against MPTP toxicity (Horvath *et al.*, 2003). Increased expression of mitochondrial uncoupling proteins protects against brain damage associated with both experimental stroke and epilepsy (Mattiasson *et al.*, 2003; Sullivan *et al.*, 2003).

CoQ₁₀ diminished ischemia-induced neuronal injury in the hippocampus (Ostrowski, 2000). CoQ₁₀ protects cultured cerebellar neurons against excitotoxin-induced degeneration (Favit *et al.*, 1992). We studied the effects of administration of CoQ₁₀ on lesions produced by mitochondrial toxins. Oral administration of CoQ₁₀ produced dose-dependent neuroprotective effects against malonate-induced striatal lesions as well as depletions of ATP and increases in lactate concentrations (Beal *et al.*, 1994). Administration of CoQ₁₀ produced significant protection against dopamine depletions induced by MPTP administration (Beal and Matthews *et al.*, 1997). Oral administration of CoQ₁₀ for 1 week prior to coadministration of 3-nitropropionic acid resulted in a significant 90% neuroprotection against 3-nitropropionic acid induced striatal lesions (Matthews *et al.*, 1998). We found that oral administration of CoQ₁₀ starting at 50 days of age significantly increased life span of ALS transgenic mice (Matthews *et al.*, 1998), and increased survival in HD transgenic mice by 14.5% (Ferrante *et al.*, 2002). Administration of CoQ₁₀ significantly delayed the development of motor deficits, weight loss, cerebral atrophy, and neuronal inclusions.

We administered CoQ₁₀ at a dose of 360 mg per day to HD patients for 1–2 months (Korozhetz *et al.*, 1997). CoQ₁₀ therapy led to a significant 37% reduction in occipital cortex lactate concentrations, which reversed following discontinuation of therapy, indicating a therapeutic effect of CoQ₁₀. A tolerability study of CoQ₁₀ in HD patients showed that there were minimal adverse effects at

doses of 600–1200 mg daily (Feigin *et al.*, 1996). In the CARE-HD trial 360 patients were treated for 30 months (The Huntington Study Group, 2000). They were randomized to CoQ₁₀ at 600 mg per day, remacemide at 600 mg per day or the combination in a 2 × 2 factorial design. The primary outcome variable was change in the Unified Huntington's Disease Rating Scale. In this trial, CoQ₁₀ slowed decline on the total functional capacity measure scale by 14% over 30 months.

The CoQ analogue idebenone reduces cardiac hypertrophy in patients with Friedreich's ataxia (Hausse *et al.*, 2002; Rustin *et al.*, 1999). A study of the effects of CoQ₁₀ in patients with Friedreich's ataxia showed improvement of cardiac and skeletal muscle bioenergetics (Lodi *et al.*, 2001). Coenzyme Q₁₀ was administered at 400 mg daily and after 3 months of treatment the cardiac phosphocreatine to ATP ratios showed a mean relative increase to 178% of initial values.

A phase II clinical trial in patients with Parkinson's disease enrolled 80 patients who were randomly assigned to placebo or CoQ₁₀ at doses of 300, 600, or 1200 mg per day (Shults *et al.*, 2002). The primary outcome measure was the Unified Parkinson's Disease Rating Scale, which was administered at screening, baseline, and 1, 4, 8, 12, and 16 months. The subjects were patients with early PD who did not require treatment (levodopa) for their disability. They were followed up for 16 months or until disability requiring treatment with levodopa had developed. The difference between the 1200-mg and placebo groups was significant with a $p = 0.04$, with an overall slowing of disability of 44% at 16 months.

ANTIOXIDANTS AND AD

A prior study showed that vitamin E has efficacy in slowing the progression of AD (Sano *et al.*, 1997). Ginkgo biloba also may exert beneficial effects (Le Bars *et al.*, 1997; Oken *et al.*, 1998). The antioxidants curcumin and melatonin exert beneficial effects on amyloid deposition in transgenic mouse models of AD (Lim *et al.*, 2001; Matsubara *et al.*, 2003). It is, therefore, possible that CoQ₁₀ might similarly be beneficial in AD.

CONCLUSIONS

There is a large body of evidence implicating both mitochondrial dysfunction and oxidative damage in the pathogenesis of AD and PD. CoQ₁₀ administration can increase brain and brain mitochondrial concentrations in brain in mature and older animals. There is substantial

evidence that CoQ₁₀ can act in concert with α -tocopherol as an antioxidant within mitochondria. CoQ₁₀ administration is neuroprotective against ischemia and lesions produced by mitochondrial toxins including malonate, 3-nitropropionic acid, and MPTP. CoQ₁₀ extends survival in a transgenic mouse models of ALS and HD. Initial clinical trials in Friedreich's ataxia, HD, and PD have shown beneficial effects. Several other antioxidants have the potential of ameliorating the progressive neurodegeneration which occurs in AD and PD. Lastly, it is possible that antioxidants may have additive or synergistic effects with agents targeting other modalities of cell death, such as apoptosis.

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Development of Mitochondrial Gene Replacement Therapy

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Many "classic" mitochondrial diseases have been described that arise from single homoplasmic mutations in mitochondrial DNA (mtDNA). These diseases typically affect nonmitotic tissues (brain, retina, muscle), present with variable phenotypes, can appear sporadically, and are untreatable. Evolving evidence implicates mtDNA abnormalities in diseases such as Alzheimer's, Parkinson's, and type II diabetes, but specific causal mutations for these conditions remain to be defined. Understanding the mtDNA genotype-phenotype relationships and developing specific treatment for mtDNA-based diseases is hampered by inability to manipulate the mitochondrial genome. We present a novel protein transduction technology ("protfection") that allows insertion and expression of the human mitochondrial genome into mitochondria of living cells. With protfection, the mitochondrial genotype can be altered, or exogenous genes can be introduced to be expressed and either retained in mitochondria or be directed to other organelles. Protfection also delivers mtDNA *in vivo*, opening the way to rational development of mitochondrial gene replacement therapy of mtDNA-based diseases.

KEY WORDS: Mitochondria; mitochondrial DNA; protein transduction; mitochondrial genome.

Known point mutations in mitochondrial DNA (mtDNA) are relatively rare and associated with a wide variety of "mitochondrial" diseases affecting brain, retina, optic nerve, muscle, heart, endocrine organs, and liver (Graff *et al.*, 2002; McFarland *et al.*, 2002; Schapira, 2000; Schmiedel *et al.*, 2003; Zeviani and Carelli, 2003). These conditions are notable for delayed expression of variable phenotypes, and the underlying mechanisms of cellular pathophysiology remain unclear. Because the mitochondrial genome codes for only 13 out of the ~90 electron transport proteins and the hundreds or probably >1000 of all mitochondrial proteins, it remains challenging to formulate how one or more mutations in this small genome can have such profound physiological effects. In addition, mtDNA deletions accumulate with aging and may contribute to bioenergetic failure of older muscle fibers and neurons, resulting in sarcopenia and degenerative diseases such as Alzheimer's and Parkinson's (Wallace, 2001). In

all of these conditions, understanding the dynamics of mitochondrial genome replication and expression in individual cells will provide insight into disease pathophysiology. For those conditions where mitochondrial genome mutations are causal for disease expression, supplementation with normal mitochondrial genomes, or ideally replacement of defective with normal mitochondrial genomes, has great therapeutic potential. However, carrying out these critical studies has been hampered by limitations in manipulating *in situ* the mitochondrial genome inside mitochondria of living cells.

We have recently developed novel technologies to remove and replace the human mitochondrial genome inside mitochondria of human cells. Using lambda phage virus as a transfection vector and lambda phage receptor targeted to mitochondria, we demonstrated that the entire human mitochondrial genome with an inserted mitochondrial-specific GFP reporter can be transfected into ρ_0 mitochondria (mitochondria without any mtDNA) of cells. In this process ("mitofection"), mtDNA replication, mitochondrial GFP expression, and restoration of bioenergetic function occur rapidly over several days (Khan *et al.*, in preparation). Subsequently, the original "mitofection" technology has been significantly improved by the development of an engineered protein transduction system

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to transport mtDNA across cell membranes and target it to mitochondria. This technology ("protofection") introduces mtDNA into mitochondria within minutes, restores bioenergetic activity of ρ_0 cells within 1–2 days, and is active in vivo in animals. Protofection can be used to deliver the entire normal mitochondrial genome, or PCR-generated fragments, mutations, or deletions. Third, silencing the mtDNA polymerase (POL- γ) by RNA interference results in complete loss of detectable POL- γ activity and detectable mtDNA within 72 h with recovery of activity in 5–7 days. This allows creation of ρ_0 cells quickly without the use of mutagens or reverse transcriptase inhibitors and is applicable to nondividing cells such as neurons. The combined use of RNA interference to silence genes critical for mtDNA replication, and "protofection" to introduce healthy mitochondrial genomes into mitochondria of living cells, sets the stage for the realistic possibility for mitochondrial gene therapy of a wide variety of conditions.

A GENERAL INTRODUCTION TO MITOCHONDRIA AND THEIR GENES

Although the origins of modern mitochondria are not known with certainty, the endosymbiotic theory proposed originally by Margulis (Margulis, 2001) remains one of the most cogent. In this construct, modern mitochondria developed from bacterial precursors who inhabited early prokaryotes and provided a mechanism to detoxify oxygen. These bacterial invaders established a complex symbiotic relationship with their hosts that included improved metabolic efficiency and sharing of genomic responsibilities, with gradual transfer of mitochondrial genomic responsibilities to the host nucleus (Gray *et al.*, 2001). Early in evolution, the mitochondrial genome of eukaryotes thus shrank from its large bacterial predecessor to a much-reduced size (366.9 kB in *A. thaliana*; 85.8 kB in *S. cerevisiae*; 13.8 kB in *C. elegans*; 16.5 kB in *H. sapiens*). The human mitochondrial genome, similar to that of other mammals, is intron-less, circular, and codes for 13 electron transport proteins, 2 ribosomal RNAs and 22 tRNAs. The mitochondrial genetic code(m) is similar to, but not identical with, the nuclear code (n), differing in four codons (*AUA* = Ile (n), Met (m); *UGA* = Term (n), Trp(m); *AGA, AGG* = Arg (n), Term (m)).

The total number of mitochondrial proteins is not accurately known, but certainly numbers are in the hundreds and possibly thousands (Lescuyer *et al.*, 2003; Sickman *et al.*, 2003). The vast majority of mammalian mitochondrial proteins are thus coded by nuclear genes and targeted to mitochondria by N-terminal mitochondrial local-

ization sequences (MLS). The MLS-targeted proteins are imported into mitochondria in an energy-dependent manner by membrane translocase complexes, known as the translocase of outer membrane (TOM) and translocase of inner membrane (TIM). Following importation, MLS is removed and proteins are incorporated into electron transport chain, outer or inner membrane, intermembrane space or matrix.

The myriads of critical functions performed by mitochondria, including both the historically first described role of respiration and ATP synthesis, and now including the participation in calcium signaling and buffering and control of cell death activation (Ganitzkevich, 2003; Hajnoczky *et al.*, 2003a, 2003b; Orrenius *et al.*, 2003; Smaili *et al.*, 2003; Vandecasteele *et al.*, 2001), require coordinated expression and stoichiometrically regulated importation and incorporation of the hundreds (at least) of nuclear genome-encoded proteins of diverse functions and expression of 13 mitochondrial genome-encoded proteins devoted to electron transport chain function. Considering the complexity of this critical organelle, it is remarkable that it consistently is assembled in working order, repaired efficiently, and passed on regularly to progeny cells.

TRANSDUCTION DOMAINS FOR DELIVERY OF THERAPEUTIC PROTEINS

The blossoming field of genomics, through utilization of advanced transfection protocols and gene microarrays, is leading researchers to the discovery of many novel therapeutic proteins. However, delivery of these large proteins across cell membranes, into senescent cells and across the blood–brain barrier (BBB), presents a substantial hurdle to utilizing this method of therapeutic intervention. With the introduction of Protein Transduction Domain- (PTD) Protein fusions, the hurdle is diminishing and appears to be increasingly surmountable. These small regions of proteins are able to cross the cell membrane in a receptor-independent mechanism.

Although several of these PTDs have been documented, the two most commonly employed PTDs are derived from the TAT protein from HIV and Antennapedia transcription factor from *Drosophila*, whose PTD is known as Penetratin (Derossi *et al.*, 1994).

TAT protein consists of 86 amino acids and is involved in the replication of HIV-1. The TAT PTD consists of an 11 amino acid sequence domain of the parent protein that appears to be critical for uptake (Vives *et al.*, 1997). In the current literature TAT has been favored for fusion to proteins of interest for cellular import. Several modifications to TAT, including substitutions of glutamine to alanine (Q \rightarrow A), have also demonstrated an increase in

cellular uptake anywhere from 90% (Wender *et al.*, 2000) to up to 33-fold (Ho *et al.*, 2001) in mammalian cells.

PROPERTIES OF PROTEIN TRANSDUCTION DOMAINS

Highly Efficient Uptake

Intracellular delivery of various therapeutic proteins involving TAT-PTD fusions has proven to be quite effective. This type of fusion protein was recently utilized in the delivery of biologically active antioxidant enzymes such as catalase (CAT). When exposed to H₂O₂, HeLa cells demonstrated a 90% increase in cell viability as compared to controls (Jin *et al.*, 2001).

Kinetic studies on the uptake of PTD have shown that an entire cell population can reach maximum uptake of PTD within as little as 30 s to 5 min of initial exposure (Ho *et al.*, 2001). PTDs provide for rapid uptake of attached proteins, although these fusion proteins can vary in uptake in a tissue-specific manner and also depend on the structure and size of the protein fused.

Stability of transduced fusion proteins into cultured HeLa cells demonstrated a peak concentration at approximately 2 h of incubation with a steady decrease up to 72 h later (Jin *et al.*, 2001). Tat-PTD has also been fused to Angiotensin II type I receptor (AT1R) to investigate Tat-PTD fusion's transduction efficacy and functionality in neurons. Neuronal cultures isolated from the hypothalamus and brain stem of 1-day-old Wistar-Kyoto rats (WKY) were incubated with 300 μ g/mL of the recombinant protein and peak fluorescence was noted after 30 min of incubation with initial fluorescence recorded within minutes (Hammond *et al.*, 2001). These are just a few of the many examples of PTD-linked proteins that demonstrate the ability of PTD to rapidly transduce cells.

PTD Fusion Proteins Allow Delivery of Large Cargo Across BBB

Viral-mediated delivery of DNA for the production of proteins is a potentially promising technology, but it is not well suited for certain conditions, as the delivery of genes via viral vector systems is time-consuming and often presents problems of immunogenicity. Protein synthesis can also be downregulated in areas of the brain which have undergone insult, such as ischemia, as well as having undergone pathophysiological change, as is seen in MELAS brain.

Another problem with the therapeutic delivery of proteins to neuronal tissues is BBB. BBB is composed of spe-

cialized endothelial cells and tight junctions, which make delivery of even low-molecular-weight proteins, such as NGF (26-kDa dimer), a very difficult and low-efficiency process.

Protein transduction domains present a new and exciting approach to the delivery of biologically active proteins across BBB. Kilic *et al.* (2003) recently demonstrated the ability of a Tat-GDNF (Glial cell line-derived neurotrophic factor) protein to cross BBB. Delivery of Tat-GDNF fusion prevented both apoptotic and necrotic injury after short- and long-term ischemia in rats. The method of application for the Tat-GDNF recombinant protein was intravenous infusion—requiring no surgical interventions.

Cao *et al.* (2002) further demonstrated the ability of PTD proteins to cross BBB, utilizing a Bcl-xL PTD fusion. The aim of this study was to introduce Bcl-XL, a known neuronal antiapoptotic factor, to provide neuroprotection during ischemia in the murine model of focal ischemia/reperfusion. Intraperitoneal injection of PTD-HA-Bcl-xL into mice demonstrated the ability of fusion proteins to cross BBB. The protein fusion was able to decrease cerebral infarction up to 40% upon initiation of cerebral ischemia (Cao *et al.*, 2002).

A similar study utilized a Bcl-x mutant (FNK), with increased antiapoptotic activity, to protect SH-SY5Y neuroblastoma cells in vitro when exposed to staurosporine-induced apoptosis and glutamate-induced excitotoxicity. This PTD-FNK fusion was also injected i.p. into gerbils and prevented delayed neuronal death in the hippocampus caused by transient global ischemia (Asoh *et al.*, 2002).

Cytotoxicity and Immunogenicity

A key requirement for any therapeutic intervention with a PTD fusion protein is that no untoward changes in normal cell physiology or function occur. Brain microvascular endothelial cells (BMEC) exposed to Tat demonstrate marked increased levels of cellular oxidative stress, decreased levels of intracellular glutathione, and activated DNA binding activity and transactivation of NF- κ B and AP-1 (Toborek *et al.*, 2003).

The protein transduction domain utilized by us is an 11 amino acid sequence that represents a poly-Arginine stretch shown to be higher in transduction efficiency than PTD of the Tat-HIV-1 protein. Although the 11 amino acid PTD sequence is similar to the small motif of the parent Tat-HIV-1 protein, the concern that it may be sufficient to elicit similar cytotoxicity when introduced into cell culture or animal models is noteworthy and has been addressed in my numerous publications since the discovery of PTDs. The literature to date indicates that the Tat-PTD can transduce proteins of interest to nearly 100%

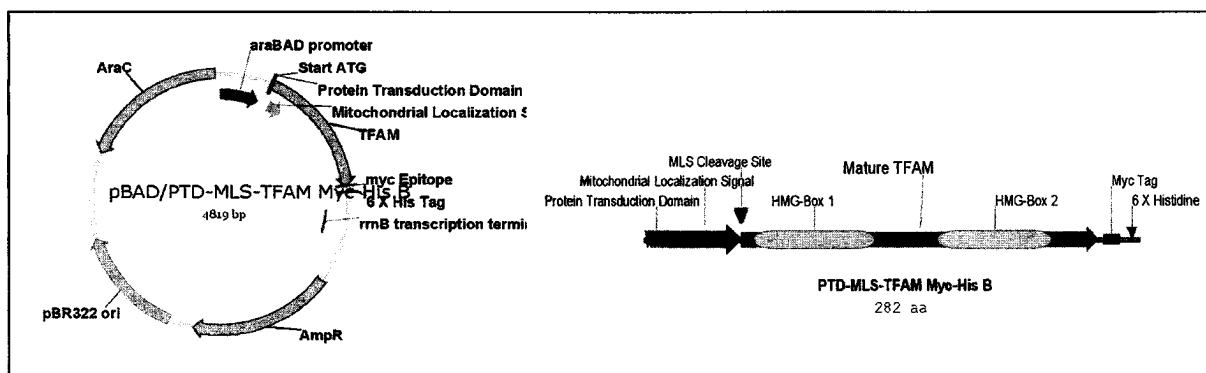


Fig. 1. Plasmid design (left) and protein structure (right) for TFAM with a PTD domain followed by an MLS.

of a cell population without exhibiting cytotoxic effects. Many groups, such as Cao *et al.* (discussed in capability of PTD to cross BBB), have also gone so far to prove the therapeutic benefits and the cell-protective capabilities that PTD-linked proteins possess. No cytotoxicity was reported upon treatment with the PTD fusion (Dolgilevich *et al.*, 2002). Jin *et al.* (2001) utilized a Tat PTD-linked SOD (super oxide dismutase) and a Tat PTD-linked hCat (human catalase) to demonstrate that the transduced fusion proteins remained enzymatically stable for 60 h. The fusion protein did not elicit any cellular toxicity and was able to increase HeLa cell viability up to 90% upon exposure to H_2O_2 . Leifert *et al.* (2002) also recently reported that full-length proteins attached to the HIV-Tat protein transduction domain neither are transduced between cells nor exhibit enhanced immunogenicity. These experiments, as well as many others in the literature to date, demonstrate the potential therapeutic efficacy of PTD-linked proteins with no found toxicity or increased immunogenicity of the fusion proteins.

Mitochondrial Localization of PTD-Fusion Proteins

Del Gaudio and Payne (2003) have characterized the feasibility of using protein transduction to target proteins to mitochondria. They found that a mitochondrial localization signal was necessary to enable persistence of the targeted protein inside mitochondria. Also, neither loss of the mitochondrial import machinery nor decrease in $\Delta\Psi M$ inhibited entry and retention of their fusion protein. Finally, in pregnant mice injected with their TAT-mMDH-GFP fusion protein, the protein crossed the placenta and was found in fetal and neonatal pups, indicating that the protein not only crossed multiple membrane barriers but also persisted within mitochondria.

TFAM IS A MITOCHONDRIAL HISTONE

Mitochondrial transcription factor A (TFAM) is a 246 amino acid (~25 kDa) protein first isolated and cloned as a transcription factor for mtDNA (Fisher and Clayton, 1988). It is a member of the High Mobility Group (HMG) of proteins, contains two HMG domains and a 42 amino acid mitochondrial localization sequence, binds to ~25 bp of mtDNA, and is capable of bending and unwinding mtDNA (Parisi *et al.*, 1993; Parisi and Clayton, 1991). Several important TFAM binding regions on mtDNA have been identified, and endogenous mtDNA is bound to ~1000-fold molecular excess of TFAM (Alam *et al.*, 2003). TFAM is critical for mtDNA replication (Larsson *et al.*, 1998) and is controlled by transcription factors such as NRF-1 and NRF-2 known to regulate mitochondrial biogenesis (Choi *et al.*, 2004).

Figure 1 shows the plasmid construction for creation of the recombinant PTD-MLS-TFAM and the resulting molecule. Figure 2 shows successful introduction into

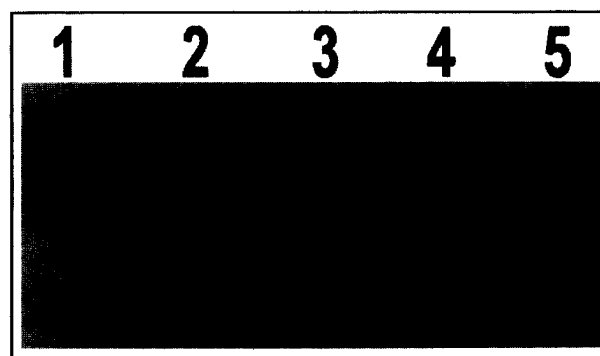


Fig. 2. Agarose gel of PCR products amplifying region around LHON 11778A mutation after SfaN1. Lane 1—LHON Cybrid; Lane 2—Sy5y; Lane 3— ρ_0 ; Lane 4—LHON mtDNA Prototected ρ_0 ; Lane 5—Prototected ρ_0 no DNA.



Fig. 3. Time course of Alexa 488 labeled MtDNA complexed with PTD-MLS-TFAM added to Sy5y cells. Red = Mito Tracker Red.

and replication of LHON mtDNA in ρ_0 cells. The LHON 11778A mutation causes loss of the SfaN1 site present in w.t. mtDNA. In ρ_0 cells a similar w.t.-like pseudogene is amplified and cut by SfaN1. Following protofection of LHON mtDNA into ρ_0 and passage through metabolic selection, mainly the introduced LHON mtDNA free of the SfaN1 site is found. Figure 3 shows that w.t. mtDNA that has been labeled with Alexa 488 dye and complexed with PTD-MLS-TFAM rapidly enters mitochondria of SY5Y cells and is concentrated within 15 min. Figure 4 shows

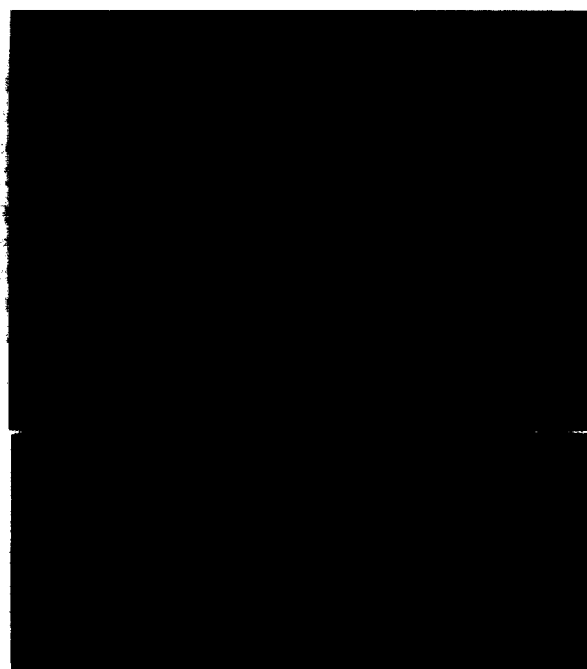


Fig. 4. MtRed and BrdU (FITC) staining of ρ_0 (A), normal SY5Y (B) and ρ_0 16 h after protofection with mtDNA complexed with PTD-MLS-TFAM (C).

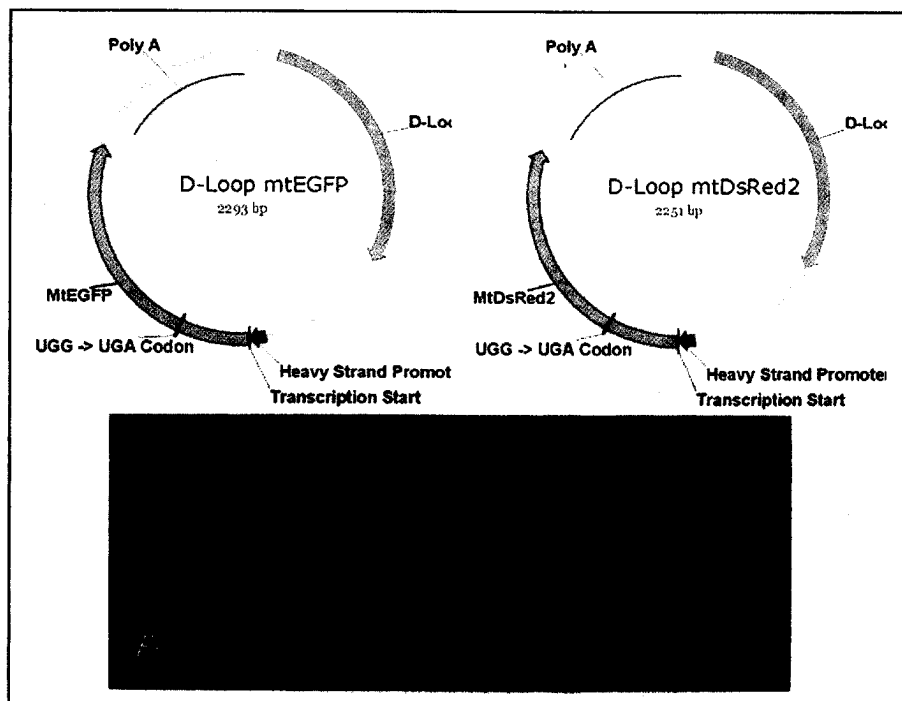


Fig. 5. (top) Constructs for generating D-loop MtEGFP (left) and D-loop MtDsRed2 (right). (bottom). Normal SY5Y cell (A) and human cortical neuron (HCN, B) 24 h after Protofection with D-loop MtEGFP construct and counterstained with Mito Tracker Red.

the rapid restoration of mtDNA replication and bioenergetic function following introduction of w.t. mtDNA by protofection. The top image (A) is of ρ_0 cells stained with Mito Tracker Red (MTRed), to localize mitochondria as a function of their $\Delta\Psi M$, and following incubation for 12 h with BrdU and immunostained for BrdU with FITC. Note the low levels of MTRed accumulation, reflecting low $\Delta\Psi M$, and absence of BrdU staining. Part (B) shows a normal SY5Y cell and part (C) shows a ρ_0 cell 16 h after protofection with PTD-MLS-TFAM complexed with w.t. mtDNA. Note the marked increase in MTRed uptake and BrdU staining.

EXPRESSION OF EXOGENOUS GENES IN MITOCHONDRIA USING PROTOFECTION

Because of our desire to be able to express individual genes inside mitochondria, in addition to the entire mitochondrial genome, we have pursued using Protfection technology to deliver small reporter genes directly to mitochondria. The premise behind the constructs is to place a gene of interest downstream of the mitochondrial D-loop and heavy strand promoter and incorporate a polyA tail. The constructs for D-loop-MtEGFP and D-loop-MtDsRed2 are shown in Fig. 5. Recall that the two reporter fluorescent proteins have been mutated so as to be specific for the mitochondrial translation apparatus. Figure 5 also shows a normal SY5Y cell (A) and a human cortical neuron (B) 24 h after Protfection with D-loop MtEGFP. There is robust mitochondrial GFP signal in mitochondria of SY5Y and several small areas of EGFP signal in the human cortical neurons.

PROTOFECTION TECHNOLOGY AND MITOCHONDRIAL GENE THERAPY

The above results show that PTD-MLS-TFAM protfection technology provides a rapid and efficient approach to providing "healthy" mitochondrial genomes to cells. Theoretically, mitochondrial diseases with homoplasmic mutations can be treated by dilution of the pathogenic mitochondrial genomes with healthy ones. Exogenous individual genes of interest can also be introduced into mitochondria, and with the appropriate codon changes, can be restricted to mitochondrial translation.

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Mitochondrial Degeneration in Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that causes motor neuron degeneration, progressive skeletal muscle atrophy, paralysis, and death. To understand the mechanism of motor neuron degeneration, we have analyzed the clinical disease progression and the pathological changes in a **transgenic mouse** model for ALS. We found massive mitochondrial vacuolation at the onset of disease. **By detailed** morphological observations, we have determined that this mitochondrial vacuolation is developed from expansion of mitochondrial intermembrane space and extension of the outer membrane and involves peroxisomes. Lysosomes do not actively participate at all stages of this vacuolation. We conclude that this mitochondrial vacuolation is neither classical mitochondrial permeability transition nor autophagic vacuolation. Thus, this appears to be a new form of mitochondrial vacuolation and we term this as mitochondrial vacuolation by intermembrane space expansion or MVISE.

KEY WORDS: Mitochondria; mitochondrion; amyotrophic lateral sclerosis; motor neuron; motoneuron; spinal cord; neurodegenerative disease.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an age-dependent neurodegenerative disease that causes motor neuron degeneration, progressive skeletal muscle atrophy, paralysis, and death (Rowland and Shneider, 2001). The etiology in most cases is not clear. Studies have implicated redox imbalance, protein aggregation, cytoskeleton disorganization, defective axonal transport, and chronic ischemia playing a role in motor neuron death (Cleveland and Rothstein, 2001). Environmental toxins and lack of dietary vitamin E have also been suggested to cause this

disease (Cox *et al.*, 2003; De la Rua-Domenech *et al.*, 1997).

One crucial question in understanding the disease mechanism is how these different noxious assaults lead to motor neuron degeneration. Are there common, converging cellular pathways? Early studies on humans were limited to pathological examination of autopsy specimens. Those studies revealed gigantic neurofilament swellings in proximal axons (Carpenter, 1968). For some years the neurofilament abnormalities had been the focus for studying the disease mechanism (Hirano, 1991).

In 1993, the first genetic cause for ALS was discovered (Rosen *et al.*, 1993). Mutations in Cu, Zn superoxide dismutase (SOD1) cause a subset of familial ALS. Soon after, several animal models for ALS were constructed by expressing the disease-associated SOD1 mutants in transgenic mice (Bruijn *et al.*, 1997; Gurney, 1994; Ripps *et al.*, 1995; Wong *et al.*, 1995). These transgenic mice have allowed studies of motor neuron degeneration process in unprecedented detail. It is from these studies that mitochondrial degeneration and dysfunction have emerged as an important early event in motor neuron degeneration process. This review summarizes efforts from our laboratory in characterizing mitochondrial degeneration in an ALS animal model. Readers who are interested in learning

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more about mitochondrial degeneration in ALS are referred to several broad reviews that are published recently (Julien, 2001; Menzies *et al.*, 2002b).

MITOCHONDRIAL DEGENERATION IS AN EARLY EVENT IN MOTOR NEURON DEGENERATION PATHWAY

Early observations on mutant SOD1 transgenic mice reported mitochondrial vacuolation as well as neurofilament accumulation in motor neurons (Dal Canto and Gurney, 1995; Julien, 2001; Wong *et al.*, 1995). The neurofilament pathology was expected but the severe mitochondrial abnormalities were a surprise. Were these pathologies revealing crucial steps in motor neuron degeneration pathway or were they simply representing by-products of degenerative process—a mere consequence of cellular degeneration? This question motivated us to conduct a systematic study. We reasoned that abnormalities that occurred early in the disease process were more likely to contribute to the progression of the degeneration while the abnormalities that appeared late in the disease stages, when substantial neuronal death had already been well under way, were more likely to be a consequence of early degenerative process. The early events are also more interesting in terms of therapeutic intervention, because stopping disease progression early in its track enhances the probability of motor neurons being rescued. Thus, a study that correlates the clinical progression and the sequence of pathological events (the kind of study that is impossible to conduct in humans) would be particularly revealing.

We conducted our studies in a low expresser line of mutant SOD1G93A that was generously made available by Gurney *et al.* (1994). Because the onset and progression of the disease in the mutant SOD1 transgenic animals were heterogeneous, we first used a behavioral assay to determine the process of clinical disease progression. By this assay the disease progression was divided into four stages according to the relative muscle strength measured from the mice: a premuscle weakness (PMW) stage during which the muscle strength remained steady in mutant mice and was indistinguishable from wild-type mice; a rapid declining (RD) stage during which the muscle strength declined suddenly and precipitously; a slow declining (SD) stage during which the muscle strength declined gradually in a prolonged period; and finally the paralysis stage during which one or multiple limbs became totally immobile (Kong and Xu, 1998; Fig. 1). Interestingly, similar patterns of clinical progression, particularly the rapid decline of muscle strength at the onset of ALS, have

also been reported in human longitudinal observations (Aggarwal and Nicholson, 2002; Kasarskis and Winslow, 1989).

By collecting tissues from mice at different disease stages, we studied populations of mutant SOD1 transgenic mice synchronized for their disease stages. These studies revealed several surprises. First, at the RD stage when the disease began (60–90 days before paralysis), the loss of motor neurons was minor (less than 10%). The largest loss of motor neuron occurred at the paralysis stage (Fig. 1). This pattern correlated with the changes in astrogliosis, which also sharply rose at the paralysis stage (Levine *et al.*, 1999; Fig. 1). This suggested that early therapeutic intervention after onset of ALS may rescue the majority of motor neurons. Second, deliberate searching for prominent neurofilament abnormalities revealed few sites of neurofilament accumulation. These minor changes occurred mostly in late SD and paralysis stages. In contrast, widespread vacuoles in the spinal cord were easily observed without deliberate searches. By quantitative measurements, the number of these vacuoles peaked at the RD stage. As the disease further progresses towards the paralysis stage, the number of vacuoles declined (Kong and Xu, 1998; Fig. 1).

The peaking of vacuoles in the ventral horn was particularly interesting because it represented a dominant early pathological event. The follow-up detailed microscopic observations confirmed that these vacuoles were derived from vacuolated mitochondria (Kong and Xu, 1998). Tracing back before the onset of the disease and massive mitochondrial vacuolation, we found a large number of abnormal mitochondria associated with neuronal processes, predominantly in dendrites (Kong and Xu, 1998). This result indicated that neuronal mitochondrial damage began early, prior to the clinical onset of ALS. Indeed, functional measurement of mitochondrial complex I activity detected a decline at age 60 days, the earliest age that we measured (Jung *et al.*, 2002; Fig. 1).

MITOCHONDRIAL VACUOLATION BY INTERMEMBRANE SPACE EXPANSION (MWISE)

How do mitochondria become vacuolated in the SOD1G93A mutant mice? Our electron microscopic observations suggested a pattern of progression in mitochondrial vacuolation (Higgins *et al.*, 2003; Fig. 2). Initially mitochondria are swollen and their cristae are disorganized. But they maintain the general structure of a mitochondrion. Then the outer membrane folds at a focal point, forming a small protrusion on the mitochondrial

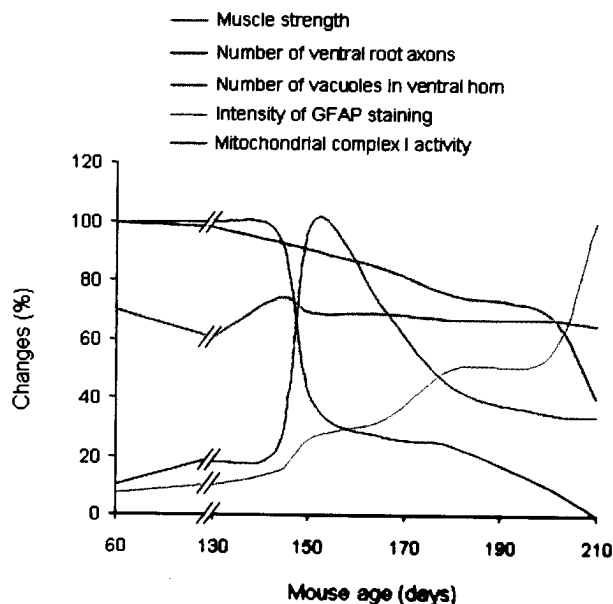


Fig. 1. Sequence of pathological events leading to motor neuron death in mutant SOD1G93A mice. This plot is based on data in Kong and Xu (1998), Levine et al. (1999) and Jung et al. (2003).

surface and creating a small space between the outer and inner membranes. This might be caused by damage to the attachment structure between the inner and the outer mitochondrial membranes. Following the formation of this small protrusion is a further detachment between the inner and the outer membranes and expansion of the intermembrane space. As the space becomes increasingly large, the inner membrane components disintegrate, forming the inner membrane remnants inside the mitochondrial vacuole (Higgins et al., 2003; Fig. 2).

This model for mitochondrial vacuolation was plausible because several other studies have shown that both wild-type and mutant SOD1 exist in mitochondrial intermembrane space (Higgins et al., 2002; Mattiazzi

et al., 2002; Okado-Matsumoto and Fridovich, 2001; Sturtz et al., 2001) and expansion of intermembrane space had been suggested (Bendotti et al., 2001; Jaarsma et al., 2001).

To test this model, we localized markers for various mitochondrial components in the vacuoles. We took the advantage of our early finding that mutant SOD1 was present at the boundary of vacuoles (Levine et al., 1999) and marked vacuoles using anti-SOD1 antibodies. By double immunofluorescent staining, we demonstrated that the inner mitochondrial membrane marker cytochrome *c* oxidase was located with the inner membrane remnants inside the vacuole. The outer mitochondrial membrane markers, transporter of outer membrane TOM20 and TOM40, were located on the outer vacuolar membrane. Cytochrome *c*, an intermembrane space marker, colocalized with SOD1 in mitochondria at the beginning stage of the vacuolation, but disappeared when the vacuoles enlarged. The disappearance of cytochrome *c* in large vacuoles could be either due to a dilution of cytochrome *c* as the intermembrane space expands or due to leakage out of mitochondrial intermembrane space. Our observation that the outer membranes of the large vacuoles were often porous supported the latter possibility (Higgins et al., 2003).

As we further looked for other organelles that might participate in mitochondrial vacuolation, we were surprised to find abundant peroxisomes inside the vacuoles and that lysosomes were not associated with the vacuoles (Higgins et al., 2003). The features of this mitochondrial vacuolation, including the expansion of the intermembrane space (instead of mitochondrial matrix) and the presence of peroxisomes (instead of lysosomes), suggest that the mutant SOD1-induced mitochondrial vacuolation is neither the classical mitochondrial permeability transition (which involves expansion of the mitochondrial matrix) nor autophagic vacuolation (which involves lysosomes), but rather, a vacuolation by intermembrane space expansion or MVISE (Fig. 2).

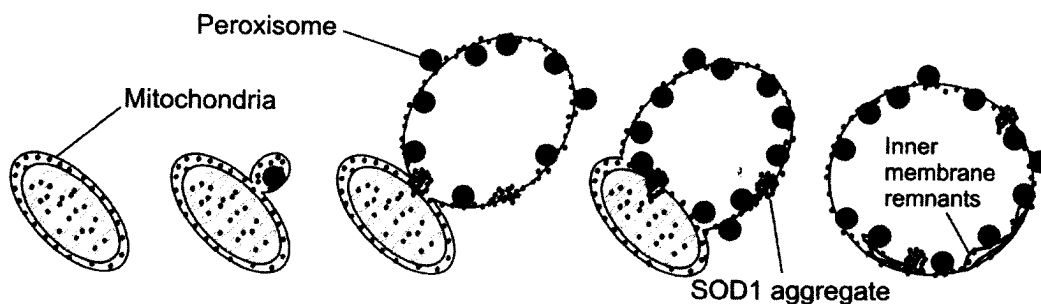


Fig. 2. Mitochondrial vacuolation by intermembrane space expansion (MVISE). This model is based on data published in Higgins et al. (2003).

THE ROLE OF MITOCHONDRIAL DEGENERATION IN MOTOR NEURON DEGENERATION PATHWAY

Experiments in cultured cells and in mice from other investigators support our *in vivo* observations on mutant SOD1-induced mitochondrial degeneration. Expression of mutant SOD1 in cultured neuroblastoma cells caused a decrease in mitochondrial membrane potential (Carri *et al.*, 1997). Similarly, expression of mutant SOD1 in motor-neuron-like cell line NSC34 cells damaged mitochondria and caused mitochondrial dysfunction and cell death (Liu *et al.*, 2002; Menzies *et al.*, 2002a). Targeting mutant SOD1 to mitochondria in cultured neuroblastoma cells produced heightened toxicity (Takeuchi *et al.*, 2002). *In vivo*, transgenic mice expressing mutant SOD1 showed a loss in mitochondrial mass (Wiedemann *et al.*, 2002). Mitochondrial toxin MTPT or a decrease in mitochondrial SOD2 activity significantly exacerbated the clinical progression of ALS in mutant SOD1 transgenic mice (Andreassen *et al.*, 2000, 2001).

Is mitochondrial degeneration relevant for human ALS? Although early studies on human autopsy focused on neurofilament accumulation in proximal axons because of their conspicuous presence, careful examination of the published EM micrographs reveals numerous vacolated mitochondria among swirls of disorganized neurofilaments in both sporadic and familial cases (Hirano *et al.*, 1984a,b). Other studies have shown vacuolated mitochondria in upper and lower motor neurons (Sasaki *et al.*, 1990; Sasaki and Iwata, 1999) as well as hepatocytes (Masui *et al.*, 1985) in human ALS cases. These observations suggest that mitochondrial degeneration play a role in human ALS.

Some SOD1 mutants cause motor neuron degeneration without mitochondrial vacuolation (Bruijn *et al.*, 1998; Ripps *et al.*, 1995; Wang *et al.*, 2002). However, whether these mutants cause functional impairment is unclear and remains a future challenge for our investigation. To further understand the role of mitochondrial damage in the motor neuron degeneration pathway, two questions need to be answered: How do SOD1 mutants damage mitochondria and what is the consequence of this damage? Mutant SOD1 may directly damage mitochondria. Both wild-type and mutant SOD1 are found in mitochondria (Higgins *et al.*, 2002; Mattiazzi *et al.*, 2002), probably in the intermembrane space (Okado-Matsumoto and Fridovich, 2001; Sturtz *et al.*, 2001). Mutant SOD1 aggregates in mitochondria and the aggregates are associated with mitochondria membranes (Higgins *et al.*, 2003). Therefore, the aggregates could cause direct damage to mitochondrial membranes. Evidence for protein aggre-

gates damaging biomembranes has emerged from studies on Parkinson's disease, where mutant α -synucleins forms aggregates and annular rings on membranes (Lashuel *et al.*, 2002), and causing permeabilization of membranes (Volles *et al.*, 2001). Mutant SOD1 could damage mitochondria by similar mechanisms.

Mutant SOD1 could also damage mitochondria indirectly. Mutant SOD1 interacts with cellular chaperones (Okado-Matsumoto and Fridovich, 2002; Shinder *et al.*, 2001) and inhibits chaperone activity in motor neurons (Batulan *et al.*, 2003; Bruening *et al.*, 1999). Mitochondrial protein import depends on cytoplasmic as well as mitochondrial chaperone activities (Neupert and Brunner, 2002; Young *et al.*, 2003). The reduced chaperone activity may impair mitochondrial function by interfering with protein import into mitochondria.

What is the consequence of mitochondrial damage? Mitochondrial dysfunction can lead to energy deficiency and ionic imbalance (Beal, 1992), elevated reactive oxidative stress and oxidative damage (Andreassen *et al.*, 2000), and increased sensitivity of neurons to excitotoxicity (Bittigau and Ikonomidou, 1997; Ikonomidou *et al.*, 1996; Kaal *et al.*, 2000; Kruman *et al.*, 1999). These effects could lead to structural damage to mitochondria, resulting in MVISE. The loss of structural integrity could trigger cell death programs by releasing pro-apoptotic proteins that reside in the mitochondrial intermembrane space, such as cytochrome *c*, AIF, SMAC/DIABLO, endo G, and Htra/Omi (Green and Evan, 2002). Because the majority of vacuoles develop in distal small dendrites (Levine *et al.*, 1999), the release of these proapoptotic molecules may not cause typical apoptotic changes in motor neuron cell bodies, such as chromatin condensation and cytoplasmic blebbing. Indeed, the typical changes are not observed by EM (Bendotti *et al.*, 2001; Guegan and Przedborski, 2003), but widespread caspase activation is detected in spinal cords (Guegan *et al.*, 2001; Guegan and Przedborski, 2003; Pasinelli *et al.*, 2000), suggesting the occurrence of a neuritic death program (Mattson and Duan, 1999).

In summary, there is strong evidence that mitochondrial degeneration plays important roles in mutant SOD1-induced motor neuron degeneration pathway. Further challenges in defining this role will be to determine whether SOD1 mutants that do not induce mitochondrial vacuolation impair mitochondrial function and the mechanism whereby all mutant SOD1 damages mitochondria. Because of the complexity of cellular environment, future breakthrough in mechanistic understanding of how mutant SOD1 damages mitochondria will most likely emerge from defined *in vitro* experimental systems.

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Brain Region-Specific, Age-Related, Alterations in Mitochondrial Responses to Elevated Calcium

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An age-related Ca^{2+} dysregulation and increased production of reactive oxygen species (ROS) may contribute to late-onset neurodegenerative disorders. These alterations are often attributed to impaired mitochondrial function yet few studies have directly examined mitochondria isolated from various regions of the aged brain. The purpose of this study was to examine Ca^{2+} -buffering and ROS production in mitochondria isolated from Fischer 344 rats ranging in age from 4 to 25 months. Mitochondria isolated from the cortex of the 25 month rat brain exhibited greater rates of ROS production and mitochondrial swelling in response to increasing Ca^{2+} loads as compared to mitochondria isolated from younger (4, 13 month) animals. The increased swelling is indicative of opening of the mitochondrial permeability transition pore indicating impaired Ca^{2+} buffering/cycling in aged animals. These age-related differences were not observed in mitochondria isolated from cerebellum. Together, these results demonstrate region specific, age-related, alterations in mitochondrial responses to Ca^{2+} .

KEY WORDS: Mitochondria; aging; calcium; reactive oxygen species; rat; mitochondrial permeability transition.

INTRODUCTION

Mitochondria serve a number of functions including maintenance of cellular bioenergetics, Ca^{2+} regulation, control of cell death cascades, and represent the major intracellular source of reactive oxygen species (ROS) (Fiskum, 2000). Although numerous studies have demonstrated increased oxidative stress and ROS production in the aged brain (Floyd and Hensley, 2002; Joseph *et al.*, 2000) the underlying cause is uncertain. The elevated oxidative damage could represent cumulative damage due to increased ROS production, a decline in antioxidant defenses, or a decrease in the degradation of oxidized proteins and lipids.

Previous studies have demonstrated an age-related increase in ROS production using rat brain homogenates (Baek *et al.*, 1999; Driver *et al.*, 2000), cortical slices (Kannurpatti *et al.*, 2004), or synaptosomal preparations (Choi, 1995). However, it is not known if the source of increased ROS production is mitochondrial in origin. To determine if alterations in mitochondria contribute to the age-related increases in ROS production and mitochondrial permeability transition (mPT), we examined mitochondria isolated from the cortex, hippocampus, and cerebellum of rats of 4, 13, and 25 months of age.

MATERIALS AND METHODS

Mitochondrial Isolation

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee and the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

This mitochondrial isolation protocol has been previously described (Brown *et al.*, in press; Sullivan *et al.*, 2000, 2003, 2004) and all procedures were performed on ice. Briefly, male Fischer 344 (F344) rats of

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3 different ages were decapitated and the brains rapidly removed. The cortices, hippocampi, and cerebellum were dissected out and separately placed in an all-glass dounce homogenizer containing five times the volume of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). The tissues were homogenized and mitochondria were isolated by differential centrifugation. The homogenate was spun twice at $1300 \times g$ for 3 min in an eppendorf microcentrifuge at 4°C and the supernatant was transferred to new tubes. The resulting supernatant was topped off with isolation buffer with EGTA and spun at $13,000 \times g$ for 10 min. The supernatant was discarded; the pellet was resuspended in 500 μL of isolation buffer with EGTA; and put under a pressure of 1000 psi for 5 min (or up to a maximum of 1200 psi for 10 min) inside the nitrogen cell disruption bomb from Parr Instrument Company (Moline, IL) at 4°C . After bursting of synaptosomes, the samples were brought up to a final volume of 2 mL with isolation buffer with EGTA, and centrifuged at $13,000 \times g$ for 10 min. The pellet was resuspended in isolation buffer without EGTA and centrifuged at $10,000 \times g$ for 10 min. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of ~ 10 mg/mL. The protein concentration was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL) by measuring absorbance at 560 nm with a Biotek Synergy HT plate reader (Winooski, VT). The respiratory activity of isolated mitochondria was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England) and only mitochondrial preparations with respiratory control ratios above 5 were used in the studies.

Mitochondrial Reactive Oxygen Species

Mitochondrial reactive oxygen species formation was measured using 2',7'-dichlorodihydro-fluorescein (DCF) diacetate (Molecular probes) as described previously with some slight modifications (Sensi *et al.*, 2003; Sullivan *et al.*, 2003). Isolated mitochondria (1 mg/mL) were placed in a thermostatically controlled, constantly stirred cuvette in a total volume of 1.5 mL containing respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl_2 , 2.5 mM KH_2PO_4 at pH 7.2) with 5 mM pyruvate, 2.5 mM malate, 25 U/mL horseradish peroxidase and 10 μM DCF. The arbitrary fluorescence units were measured using a Shimadzu RF-5301PC spectrofluorophotometer (excitation 485 nm, emission 530 nm). Each run was performed identically with a baseline reading of mitochondria in buffer followed

by a bolus of 50 μM CaCl_2 and then a bolus of 100 μM CaCl_2 for a total run time of 5 min per sample per region. The slope of DCF fluorescence was quantified for the respective conditions including baseline, after addition of 50 μM Ca^{2+} , and after 150 μM total exogenous Ca^{2+} using the Shimadzu Hyper RF software. Results are expressed as mean percent of baseline slope \pm SEM from five animals in each age group. Statistical analyses were performed using a oneway analysis of variance with the Bonferroni/Dunn post hoc analysis, $p < 0.05$.

Mitochondrial Swelling

Isolated mitochondria (1 mg/mL) were incubated at 37°C in de-energizing buffer of 150 mM KCl, 20 mM MOPS, 10 mM Tris, 2 μM ionomycin, 1 μM rotenone, and 1 μM antimycin-A as described previously (Sensi *et al.*, 2003; Sullivan *et al.*, 2003). After 5 min, calcium (or only buffer in controls) was added to mitochondria and the absorbance (540 nm) was measured. Mitochondrial swelling represents a decrease in absorbance, normalized, averaged and expressed in arbitrary units (AU). All measurements were made with a Biotek Synergy HT plate reader. This data represent six animals from each age group with three replicates per animal.

RESULTS

In this study, male F344 rats were used from three different age groups (groups, age, mean weight): young, 4 months old, 206.5 g; middle, 13 months old, 450.7 g; and aged, 25 months, 426.5 g. From each animal, total mitochondria were isolated separately from the cortex, hippocampus, and cerebellum. We observed increased rates of DCF fluorescence in response to elevated $[\text{Ca}^{2+}]$ in mitochondria isolated from the cortex and hippocampus (Fig. 1(a) and (b)), but not cerebellum (Fig. 1(c)), of aged rats as compared to younger animals. Specifically, isolated cortical mitochondria from the aged rat had significantly increased rates of DCF fluorescence as compared to the young- and middle-age groups at both concentrations of calcium. In addition, the aged hippocampal mitochondria had significantly greater rates of ROS generation than the young-age group at the 50 μM and 150 μM $[\text{Ca}^{2+}]$. Aged cortical and hippocampal mitochondria (Fig. 2(a) and (b)) underwent more rapid and larger magnitude swelling than cortical and hippocampal mitochondria from younger rats. In contrast, cerebellar mitochondria showed no age-related changes in the ROS generation (Fig. 1(c)) or mitochondrial swelling (Fig. 2(c)) across the three age groups.

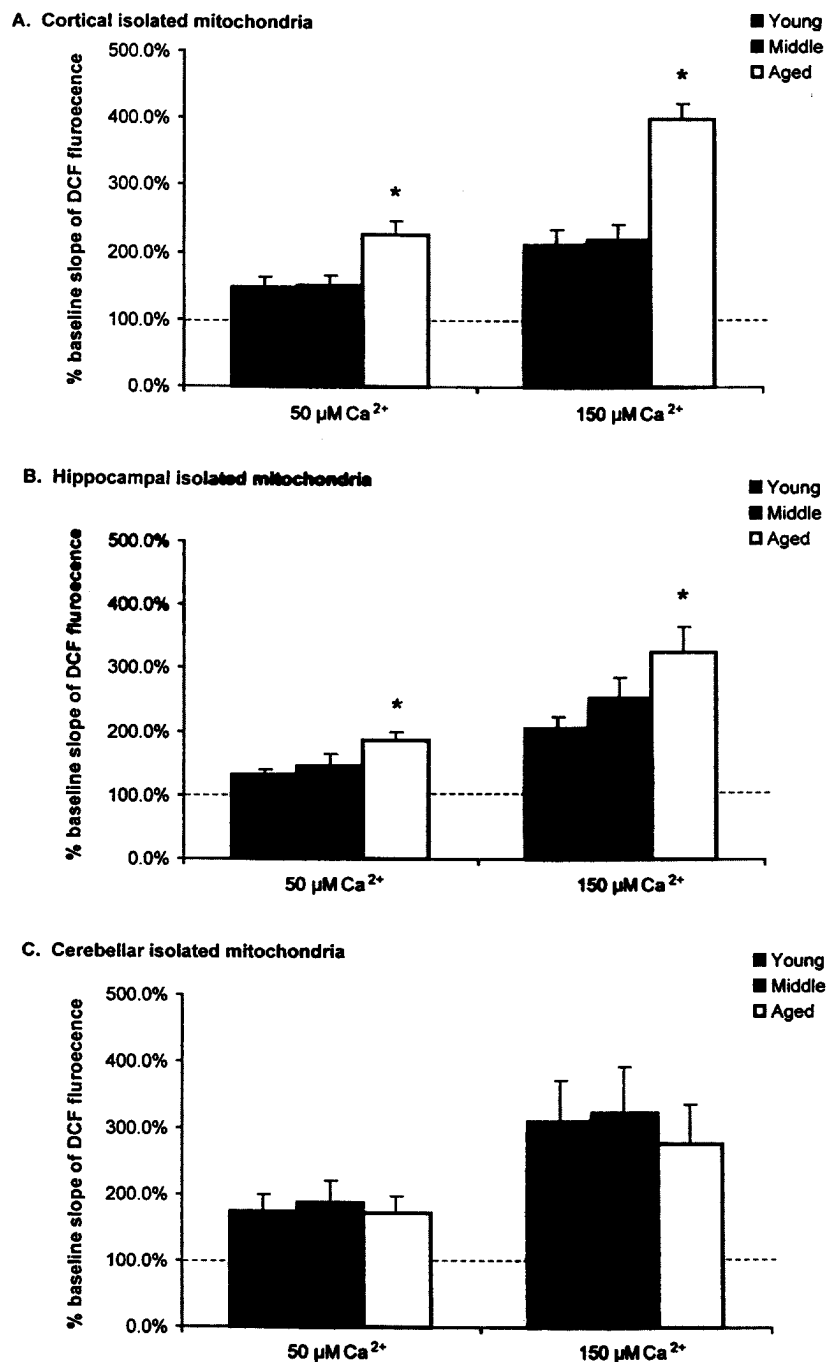


Fig. 1. Age-related differences in the rate of DCF fluorescence from three regions of the rat brain. There was an increased rate of DCF fluorescence in isolated cortical (a) and hippocampal (b) mitochondria from aged rats as compared to young- and middle-age rats after the addition of increasing concentrations of Ca²⁺ (cumulative concentration of 50 μ M and 150 μ M). However, there was no age dependant increase in the rate of DCF fluorescence in isolated cerebellar (c) mitochondria after the addition of increasing concentrations of calcium. The dotted line represents the baseline slope of DCF fluorescence (of 100%) on each graph. Statistical analyses were performed using one-way analysis of variance with Bonferroni/Dunn post hoc analysis. $p < 0.05$. Results are expressed as percent of baseline slope \pm SEM from five animals in each age group with two replicates per animal.

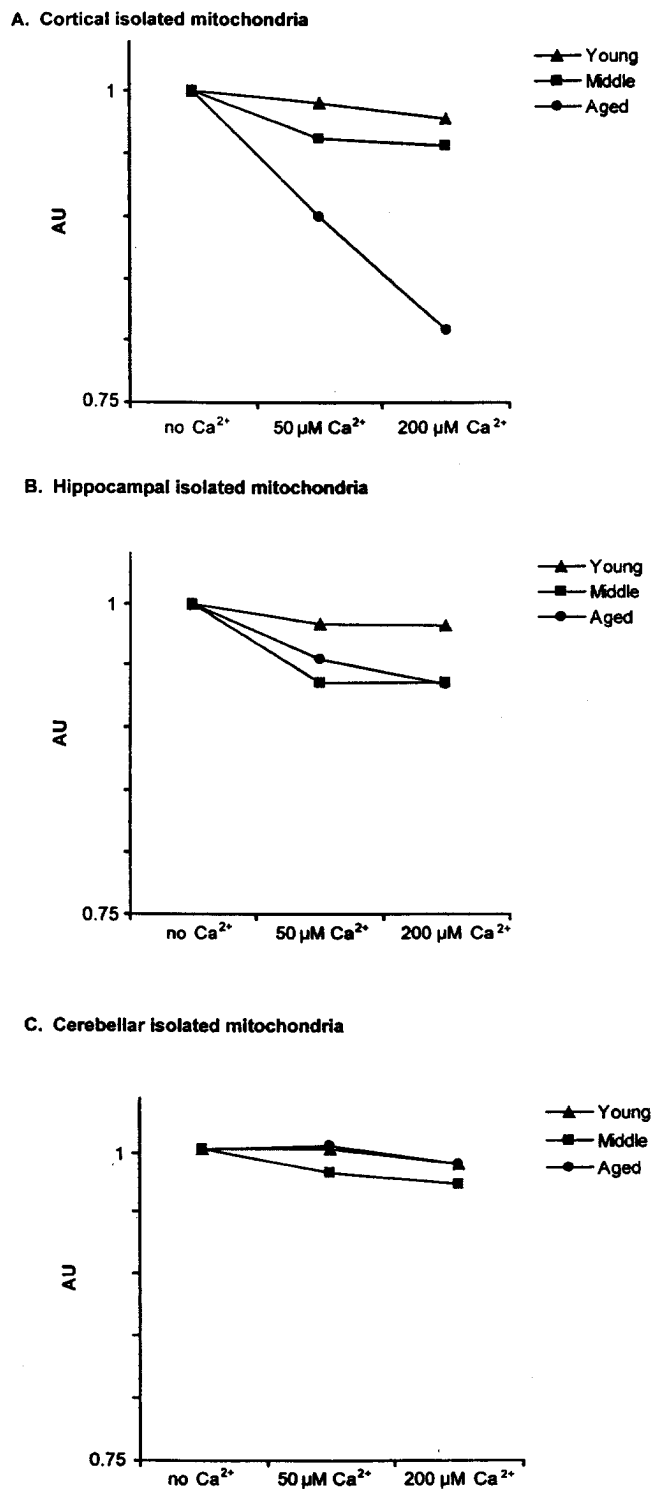


Fig. 2. Ca²⁺-induced swelling of mitochondria changes with age in isolated cortical (a) and hippocampal (b) mitochondria from F344 male rats, but not in cerebellar (c) mitochondria. Aged cortical mitochondria underwent increased swelling compared to younger rat cortical mitochondria. This data represents six animals from each age group with three replicates per animal.

DISCUSSION

The results demonstrate an increased rate of Ca^{2+} -stimulated ROS production in cortical and hippocampal, but not cerebellar, mitochondria as a function of age. The localization of the increased mitochondrial ROS production to brain regions vulnerable to late-onset neurodegenerative disorders (cortex, hippocampus) differs slightly from results obtained with homogenates in which ROS production increased with age in the cortex, but not in hippocampus or cerebellum (Back *et al.*, 1999). The mechanism(s) underlying the regional changes in Ca^{2+} stimulated mitochondrial ROS production are uncertain, but are hypothesized to reflect an age-related accumulation of oxidative damage to the mitochondria. The cortex and hippocampus both contain relatively high densities of *N*-methyl-D-aspartate receptors and may be more prone to glutamate-induced elevations in intraneuronal Ca^{2+} and the subsequent mitochondrial damage. However, the cell types in which the age-related mitochondrial alterations occurred were not examined in the present study. Current studies in our laboratory are examining ROS production in synaptic versus nonsynaptic mitochondrial fractions.

The mechanism underlying the increased rate of ROS production in response to Ca^{2+} is not clear (Galindo *et al.*, 2003; Gordeeva *et al.*, 2003; Sousa *et al.*, 2003). A previous view was that Ca^{2+} influx uncoupled the proton gradient from ATP synthase, resulting in increased flux through the electron transport chain and therefore increased ROS production. However, proton ionophores or chemical uncouplers such as FCCP result in decreased mitochondrial ROS production (Hansford *et al.*, 1997; Sullivan *et al.*, 2003). Recent data suggest that Complex I is a major source of ROS production (Liu *et al.*, 2002), either through succinate oxidation and reverse electron flow from Complex II or via the oxidation of NADH-dependent respiratory substrates by Complex I (Starkov and Fiskum, 2003). Importantly, in our studies we utilized the NADH-linked substrates malate and pyruvate to mimic more physiologically relevant conditions than succinate driven respiration. By inducing mitochondrial swelling and the release of cytochrome *c* from mitochondria, Ca^{2+} may disrupt the efficiency of electron transport and thereby elevate ROS production. Mitochondrial nitric oxide synthase (mtNOS) is another likely source of ROS/RNS in response to Ca^{2+} . Importantly, the DCF used to measure ROS production in this study and many other studies also reacts with reactive nitrogen species (Rao *et al.*, 1992). There is evidence of a mitochondrial, Ca^{2+} -dependent, nitric oxide synthase that is distinct from the brain or neuronal NOS isoform (Ghafourifar and Richter, 1997; Lacza *et al.*, 2003) although nistic inten-

tions (Gao *et al.*, 2004). We are currently investigating the contribution of NOS activity to the Ca^{2+} -induced elevation of DCF fluorescence using mitochondria-enriched preparations.

Although neurons are largely maintained in the aging brain, they become less capable of regulating intracellular Ca^{2+} , display evidence of increased oxidative stress, and contain damaged and dysfunctional mitochondria. These alterations have each spawned their own theories of aging including the "calcium hypothesis" (Khachaturian, 1987; Landfield, 1987; Thibault *et al.*, 1998), the "free radical/oxidative stress" hypotheses (Ames and Shigenaga, 1992; Butterfield *et al.*, 1999; Harman, 1973), and the "damaged mitochondria" hypothesis (Fleming *et al.*, 1982; Kowald, 2001; Nicholls, 2002). These hypotheses are interrelated, making it difficult to differentiate between cause and effect. For example, mitochondrial Ca^{2+} levels are closely linked to free cytosolic Ca^{2+} , and any condition that elevates cytosolic Ca^{2+} would likely increase mitochondrial Ca^{2+} and ROS production. Therefore, it was not clear if age-related increases in ROS production were the result of mitochondrial or nonmitochondrial mechanisms. The results of the present study however clearly demonstrate that mitochondria isolated from the cortex and hippocampus of aged rats have greater rates of ROS production in response to elevated Ca^{2+} than do mitochondria isolated from young animals. Additionally mitochondria isolated from older animals swell in response to much lower levels of Ca^{2+} than mitochondria from younger animals. These data would indicate that age-related changes in mitochondrial function to Ca^{2+} could contribute directly to cellular Ca^{2+} dysregulation and increased oxidative stress associated with senescence.

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The Nonimmunosuppressive Cyclosporin Analogs NIM811 and UNIL025 Display Nanomolar Potencies on Permeability Transition in Brain-Derived Mitochondria

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Cyclosporin A (CsA) is highly neuroprotective in several animal models of acute neurological damage and neurodegenerative disease with inhibition of the mitochondrial permeability transition (mPT) having emerged as a possible mechanism for the observed neuroprotection. In the present study, we have evaluated two new nonimmunosuppressive cyclosporin analogs NIM811 (Novartis) and UNIL025 (Debiopharm) for their ability to inhibit mPT in rat brain-derived mitochondria. Both NIM811 and UNIL025 were found to be powerful inhibitors of calcium-induced mitochondrial swelling under energized and deenergized conditions, and the maximal effects were identical to those of native CsA. The potencies of mPT inhibition by NIM811 and UNIL025 were stronger, with almost one order of magnitude higher potency for UNIL025 compared to CsA, correlating to their respective inhibitory action of cyclophilin activity. These compounds will be instrumental in the evaluation of mPT as a central target for neuroprotection in vivo.

KEY WORDS: Cell death; apoptosis; neuron; ischemia; neurodegeneration; traumatic brain injury; amyotrophic lateral sclerosis; cyclophilin; mitochondrial permeability transition; flow cytometry.

INTRODUCTION

Cyclosporin A (CsA) is an 11-amino-acid cyclic peptide of fungal origin that is currently used clinically as an immunosuppressive agent (Sandimmun[®], Novartis).

During the last decade, CsA has demonstrated potent neuroprotection in several animal models of acute neurological damage and neurodegenerative disease (Friberg and Wieloch, 2002; Keep *et al.*, 2003; Waldmeier *et al.*, 2003). Two main cellular targets have been suggested to explain the neuroprotective effect of CsA: (i) inhibition of the phosphatase calcineurin, which is generally believed to mediate its immunosuppressive properties, and (ii) the specific inhibition of the mitochondrial permeability transition (mPT) pore (Uchino *et al.*, 1995). Mitochondria are essential to the normal life of eukaryotic cells and there is now widespread agreement that mitochondria also play an active role in the biochemical signaling underlying cell death. The mechanisms behind their involvement in cell death are continually debated and remain to be fully elucidated. One pathophysiologic mechanism that has been proposed to be essential in both triggering and amplifying apoptotic and necrotic cell death is the mPT (Lemasters *et al.*, 1998; Zamzami and

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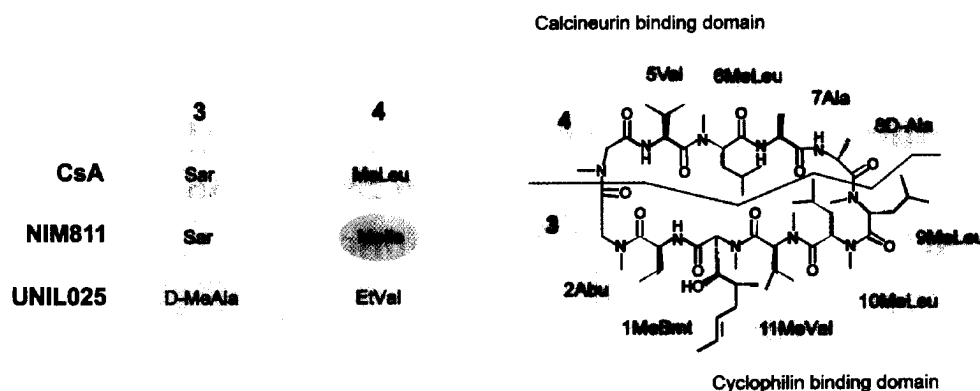


Fig. 1. Amino acid sequences for the undecapeptides cyclosporin A (CsA), NIM811, and UNIL025. The binding site for the cyclophilins to the immunosuppressive CsA molecule covers residues 1–3, 10, and 11, and the interaction with calcineurin residues 4–7. The nonimmunosuppressive cyclosporin molecules NIM811 and UNIL025 are both modified (as compared to CsA) at the fourth amino acid (from *N*-methyl-leucine to *N*-methyl-isoleucine, Melle and *N*-ethyl-valine, EtVal, respectively) preventing calcineurin binding while retaining the ability to interact with cyclophilins. UNIL025 has an additional modification at the third amino acid [from sarcosine to *N*-methyl-D-alanine (D-MeAla)]. (Modified from Waldmeier *et al.*, 2003)

Kroemer, 2001). Upon various forms of cellular stress, most noteworthy calcium overload, a proteinaceous pore in the mitochondria can open, leading to inner and outer membrane permeabilization, uncoupling of oxidative phosphorylation, mitochondrial swelling, release of reactive oxygen species and liberation of apoptogenic proteins from the intermembrane space. The mitochondrial permeability transition can be blocked by CsA, supposedly by preventing the interaction of cyclophilin D (CypD) with the adenine nucleotide translocator (ANT) and/or its complex with the voltage-dependent anion channel (VDAC) (Crompton, 1999; Halestrap *et al.*, 2002).

We have previously shown that CsA is a powerful inhibitor of mPT in de-energized and energized brain mitochondria (Hansson *et al.*, 2003, 2004). In the energized swelling paradigm the isolated mitochondria are allowed to respire on metabolic substrates and are closer to a physiological state. Under de-energized swelling conditions, the assay suffers from less interference from possible confounding factors related to the respiratory function and active ion transport mechanisms (Halestrap *et al.*, 2002). The de-energized model provides a very reliable model to study isolated pharmacological effects on mPT. Studies from our laboratory on the nonimmunosuppressive cyclosporin analog MeVal⁴-cyclosporin have demonstrated that this compound besides inhibiting mPT in brain-derived mitochondria also decreased oxygen/glucose deprivation-induced neuronal damage in cultured neurons and diminished infarct size after transient focal cerebral ischemia in rat (Friberg and Wieloch, 2002). This compound is how-

ever no longer available to researchers. The objective of the present study was to characterize the effect of two new nonimmunosuppressive (non-calcineurin inhibiting) cyclosporin analogs, NIM811 (Melle⁴-cyclosporin) and UNIL025 (MeAla³EtVal⁴-cyclosporin) (Fig. 1) on brain-derived mitochondria. As a first step in a larger project evaluating the *in vivo* neuroprotection of NIM811 and UNIL025, the compounds were examined for their ability to inhibit mPT in isolated brain mitochondria. Both classical fluorometric analyses and flow cytometry were used to determine the efficiency and potency of NIM811 and UNIL025 inhibition of mPT compared to CsA.

MATERIALS AND METHODS

Brain mitochondria were isolated from rat cerebral cortex tissue using a discontinuous Percoll gradient according to Sims, method B (Sims, 1990), with slight modifications (Hansson *et al.*, 2003). Activation of mitochondrial permeability transition was monitored by measuring the decrease in right angle light scattering at 520 nm (mitochondrial swelling) using a Perkin-Elmer spectrometer LS-50B (Emeryville, CA) or by flow cytometric detection of side scattering (SSC) using a FACSCalibur (Becton & Dickinson, San Jose, CA). The cyclosporin analogs NIM811 (Novartis, Basel, Switzerland), UNIL025 (Debiopharm, Lausanne, Switzerland) and CsA (Ivax, Opava, Czech Republic) were run under de-energized conditions to evaluate potency of mPT-inhibition,

as described previously (Hansson *et al.*, 2003). The experiments were performed at 28°C in an isotonic KCl-based buffer containing the respiratory complex blockers rotenone and antimycin (both 0.5 μ M) and the calcium ionophore A23187 (2 μ M) to ensure free diffusion of Ca^{2+} over the mitochondrial membranes. Mitochondria were incubated with 10 nM to 5 μ M of CsA, NIM811, UNIL025, or vehicle (final concentration 0.2% (v/v) ethanol) for 4 min and were subsequently exposed to 100 μ M Ca^{2+} for 5 min before termination of experiments with the ionophore alamethicin (7.5 μ g/ml).

The protocol for flow cytometric analyses of isolated brain mitochondria was described earlier (Mattiasson *et al.*, 2003). Mitochondria were analyzed for light scattering properties under de-energized conditions and selected from background on the basis of specific staining with nonyl acridine orange. Experiments were performed as described above for the fluorometric analyses but run at room temperature. The cyclosporin analogs were tested at 1 μ M and mPT was detected as a decrease in side scattering (SSC).

Inhibition of mPT by NIM811 and UNIL025 was also evaluated under energized conditions. Experiments were run as described previously in a sucrose-based buffer with 5 mM malate and glutamate as respiratory substrates, in the presence of 20 μ M ADP and 1 μ g/ml oligomycin (Hansson *et al.*, 2004). Mitochondria were incubated with the analogs at 1 μ M concentration for 3 min and then exposed to 2 μ mol Ca^{2+} /mg for 10 min before termination of experiments with alamethicin.

The decrease in light scattering from the pre- Ca^{2+} incubation value to that after alamethicin administration was defined as maximal swelling (100%). The decreases in light scattering following 5 and 10 min Ca^{2+} exposure for de-energized and energized experiments, respectively, were calculated and displayed as a percentage of maximal swelling. Swelling responses in the flow cytometric analyses were calculated in a similar fashion using the geometrical mean values (CellQuest, Becton & Dickinson, San Jose, CA). Data were analyzed with ANOVA and the Bonferroni post hoc test unless otherwise stated and presented as means \pm S.E.M.

RESULTS

The potencies of the nonimmunosuppressive analogs NIM811 and UNIL025 to inhibit calcium-induced mitochondrial swelling were evaluated under de-energized conditions, and compared to mPT inhibition by CsA (Fig. 2(A) and (B)). Means \pm S.E.M. for swelling (% max-

imal swelling; $n = 4$) were for vehicle (ethanol) $78.8 \pm 2.2\%$; and decreased in a concentration-dependent manner between $65.8 \pm 6.0\%$ for 10 nM CsA and $15.3 \pm 1.7\%$ for 5 μ M CsA; $39.5 \pm 4.9\%$ for 10 nM UNIL025 and $17.1 \pm 2.0\%$ for 1 μ M UNIL025 and $18.5 \pm 1.0\%$ for 5 μ M UNIL025; $56.6 \pm 5.9\%$ for 10 nM NIM811 to $16.8 \pm 0.5\%$ for 5 μ M NIM811 (Fig. 2B). The means for swelling were not adjusted for basal (Ca^{2+} -independent) swelling (Hansson *et al.*, 2003). Both UNIL025 and NIM811 displayed significant inhibition of swelling already at 10 nM concentration ($p < 0.0001$), 10 nM CsA was near significance ($p = 0.011$) in the stringent post hoc test used. The potency of UNIL025 was significantly higher as compared to CsA, with an approximate 10-fold difference (the 10 and 100 nM values of UNIL025 were similar to the 100 nM and 1 μ M CsA values, respectively). In Fig. 2A representative recorded traces are displayed for the lowest drug concentration tested (10 nM). In addition, CsA 1 μ M is displayed to illustrate near-maximal inhibition of swelling.

Recently, we demonstrated that flow cytometry is a useful tool to analyze mPT at the single organelle level (Hansson *et al.*, 2004). Addition of Ca^{2+} or alamethicin resulted in a decrease in side scattering (SSC) similar to that of the fluorometric decrease in light scattering described in Fig. 2A. At the pre- Ca^{2+} measurement (Fig. 2C, top), no differences in SSC between the samples were observed. Following addition of alamethicin (bottom), all samples demonstrated a strong and similar decrease in SSC (maximal swelling = 100%). Following a 5 min Ca^{2+} insult (middle), a strong decrease in SSC ($78.4 \pm 4.8\%$ of maximal swelling; $p < 0.01$, Student's *t*-test), indicative of mPT, is detected in the control sample, whereas no decreases compared to baseline values were detected in the samples treated with CsA, NIM811, or UNIL025 (9.0 ± 4.8 , 5.9 ± 2.4 , and $5.3 \pm 2.6\%$ of maximal swelling, respectively). The histograms in Fig. 2C also demonstrate that the populational spread was similar within the mitochondrial samples before and after the calcium and alamethicin insults, and that there were no subpopulations that did not respond to calcium-induced swelling (control), or that were not protected by the inhibitors of mPT (CsA, NIM811, UNIL025).

Under energized conditions, 1 μ M UNIL025 and NIM811 completely blocked mPT in isolated brain mitochondria when exposed to a calcium insult, 2.0 μ mol Ca^{2+} /mg mitochondrial protein, which produced extensive swelling in the absence of the cyclosporin analogs (Fig. 2D) Calculated values of the decrease in light scattering was slightly negative for UNIL025 ($-0.23 \pm 3.9\%$) and NIM811 ($-4.7 \pm 3.4\%$), but substantial for control ($76.2 \pm 4.1\%$) (Fig. 2E).

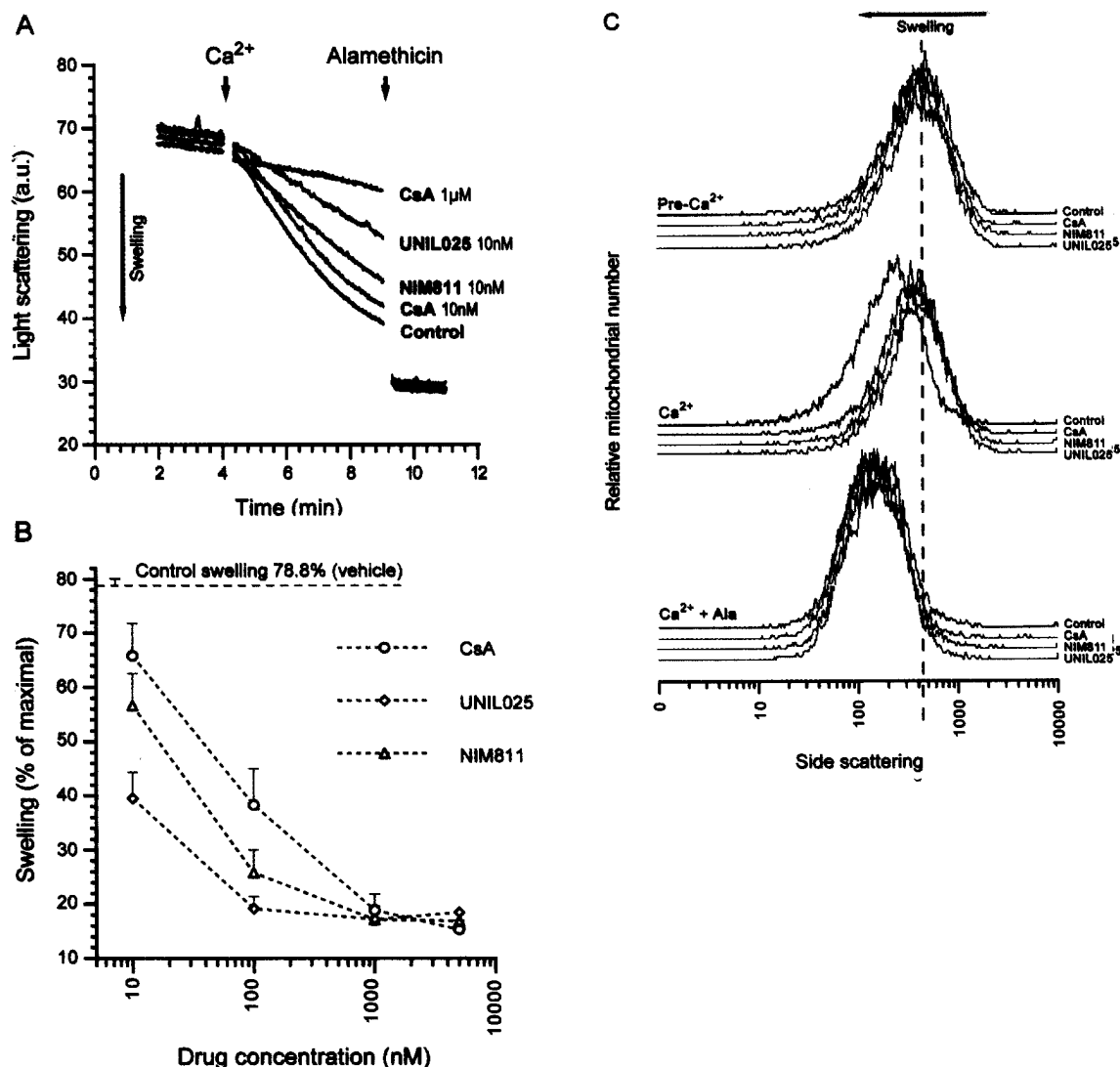


Fig. 2. The nonimmunosuppressive cyclosporin analogs NIM811 and UNIL025 inhibit permeability transition with nanomolar potencies in brain-derived mitochondria. (A) Representative traces of changes in 90° light scattering (a.u. = arbitrary units, $\Delta 520$ nm) of experiments run under de-energized conditions at 28°C in isotonic KCl-based swelling buffer, pH 7.3. UNIL025, NIM811, cyclosporin A (CsA), or vehicle (ethanol, 0.2% (v/v), control) were added to mitochondrial suspensions ($25 \mu\text{g}$ protein/ml). Ca^{2+} (final concentration $100 \mu\text{M}$) was added after 4 min and each experiment was terminated at 9 min with alamethicin ($7.5 \mu\text{g}/\text{ml}$) to induce maximal swelling. Trace disturbances due to manual mixing were deleted for clarity. (B) Calculations of concentration-dependent inhibition of de-energized swelling by CsA, UNIL025, or NIM811. Mitochondria were exposed to Ca^{2+} in the presence of 10 nM to $5 \mu\text{M}$ CsA, UNIL025, and NIM811. Mitochondria treated with Ca^{2+} in the presence of vehicle (ethanol, 0.2% (v/v), control) displayed 78.8% swelling. All values (except for 10 nM CsA, $p = 0.011$ in the stringent post hoc test used) were significantly different from control swelling. Means \pm S.E.M., $n = 4$. (C) Flow cytometric analyses of isolated brain mitochondria exposed to Ca^{2+} under de-energized conditions. The histograms show side scattering (SSC) properties of mitochondria (x-axis) and relative mitochondrial number at the respective intensities (y-axis). Samples were stained with the mitochondrial marker nonyl acridine orange, and 10 000 mitochondria were used for each measurement. SSC values were determined before Ca^{2+} (final concentration $100 \mu\text{M}$) was added to the samples (Pre- Ca^{2+} , top), following 5 min of Ca^{2+} incubation (Ca^{2+} , middle) and after induction of maximal swelling by the addition of alamethicin (Ca^{2+} + Ala, bottom). Taken together, the histograms show that Ca^{2+} -induced swelling is prevented by 1 μM CsA, NIM811, or UNIL025, and that there was no subpopulation in the samples that was not protected by these inhibitors of mPT. All histograms are representative of three individual experiments. (D) Complete inhibition by UNIL025 and NIM811 of the induction of permeability transition under energized conditions. Experiments were run at 37°C in a sucrose-based buffer containing 5 mM malate/glutamate, 1 μg/ml oligomycin, 20 μM ADP, and 1 μM UNIL025, NIM811, or vehicle (ethanol, 0.2% (v/v), control). Mitochondria ($25 \mu\text{g}/\text{ml}$) were exposed to 2 μmol Ca^{2+}/mg protein for 10 min before maximal swelling was induced by application of alamethicin. Representative traces of changes in 90° light scattering are displayed. (a.u. = arbitrary units). (E) Calculations of the extent of inhibition displayed in (D). The extent of swelling (% maximal) was calculated as described in Methods. Means \pm S.E.M., $n = 4$, *** $p < 0.0001$, one-way ANOVA followed by the Bonferroni post hoc test.

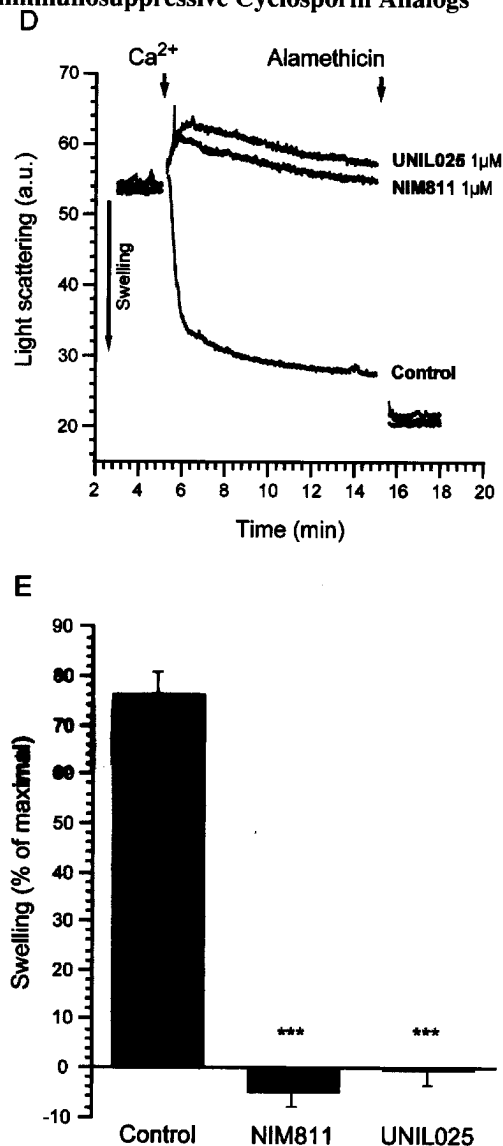


Fig. 2. Continued.

DISCUSSION

Cyclosporins have several biological activities, and their major receptors are a group of intracellular proteins known as cyclophilins. The immunosuppressive activity of CsA is generally attributed to the inhibition of the protein phosphatase calcineurin, mediated by the complex of cyclophilin A (CypA) and CsA (Borel *et al.*, 1996). The binding domains for CypA and calcineurin are located at different sites on the cyclosporin molecule (Fig. 1). Small structural alterations on the latter can dramatically reduce the binding to calcineurin while preserving high affinity to cyclophilins (Waldmeier *et al.*, 2003). A few such cyclosporins have been identified,

including the two evaluated in this study, NIM811 and UNIL025. Both are modified on the fourth amino acid, and UNIL025 has an additional modification at the third amino acid (Fig. 1). The previously available nonimmunosuppressive analog MeVal⁴-cyclosporin (PKF/SDZ 220-384, Novartis) was also modified at the fourth amino acid. Changes at residue 4 are within the critical binding site to calcineurin, and even subtle changes in the binding can largely abolish the immunosuppressive activity without reducing the cyclophilin affinity (Waldmeier *et al.*, 2003). The present study demonstrates that NIM811 and UNIL025 are equally effective at inhibiting mPT in brain mitochondria using both the de-energized and energized assays, with maximal effects similar to those of CsA (Hansson *et al.*, 2003, 2004). The dose-response studies in the de-energized model display a tendency of increased potency of NIM811 and a significant (almost 10-fold) increased potency of UNIL025 inhibiting the mPT relative to CsA. These results correlate well with the CypA binding activities of these cyclosporins. Using a competitive solid phase binding assay, NIM811 was reported to have a 1.7 times higher binding potency to CypA as compared to that of CsA (Billich *et al.*, 1995). In a cyclophilin cis-trans isomerization assay, the inhibitory potency for UNIL025 was 3.4 times that of CsA (Ruegg *et al.*, unpublished observations). The cyclosporin binding pocket of all cyclophilins is formed by a highly conserved region. Of the known cyclophilins, the mitochondrial CypD has the highest homology to the cytosolic CypA. Further, in the actual cyclosporin binding region the sequence identity is 100% between CypA and CypD (Waldmeier *et al.*, 2003). Therefore, CypA affinity is likely a very good predictor of mPT inhibition, which is dependent on the interaction between CypD and the ANT.

Mitochondria from the brain are derived from different cellular sources, glial as well as neuronal. The characteristics of mPT vary depending on the tissue and cell type from which they are derived (Bambrick *et al.*, 2004). Our previous results indicate that brain-derived mitochondria in general display CsA-sensitive permeability transition, even though they display regional differences in the sensitivity to calcium and other inducers of mPT (Friberg *et al.*, 1991; Hansson *et al.*, 2003, 2004; Mattiasson *et al.*, 2003). Here, using flow cytometry under de-energized conditions, NIM811 and UNIL025 as well as CsA displayed powerful inhibition of mPT in the whole mitochondrial population when exposed to a calcium concentration that rapidly induces mPT and large amplitude swelling when the cyclosporins were not present.

The potent inhibition of mPT by NIM811 and UNIL025 opens the opportunity to further and more stringently assess the hypothesis that mPT is involved in

neuronal cell death. Animal studies strongly implicate mPT as an important mechanism for the neuroprotection displayed by CsA. FK506, a calcineurin-inhibiting immunosuppressant that does not affect the mPT pore has generally been used as a control for calcineurin-mediated neuroprotection, and the more pronounced protective effect of CsA in certain *in vivo* and *in vitro* models has been attributed to the inhibition of mPT (for review see Hansson *et al.*, 2004; Keep *et al.*, 2003; Waldmeier *et al.*, 2003). Early results on MeVal⁴-cyclosporin has demonstrated that mPT is not dependent on calcineurin activity (Nicolli *et al.*, 1996; Petronilli *et al.*, 1994), which is further confirmed by the present data. MeVal⁴-cyclosporin has displayed neuroprotection in an animal model of focal ischemia and in a cell culture model of oxygen/glucose deprivation (Friberg and Wieloch, 2002). NIM811 has previously been demonstrated to inhibit mPT in rat liver mitochondria and to block TNF-induced apoptosis in cultured rat hepatocytes. In contrast to CsA, NIM811 was not as cytotoxic at high concentrations, implying a wider therapeutic window when inhibition of the calcineurin enzyme is absent (Waldmeier *et al.*, 2002). There are two important obstacles in translating the promising preclinical neuroprotective effects of CsA to human use. Unless the blood-brain barrier is disrupted, the penetration of CsA to the central nervous system by regular intravenous or oral administration is poor (Begley *et al.*, 1990), and immunosuppression is an undesirable side effect for many neurological indications (especially chronic diseases). The reported immunosuppressive activity of NIM811 is 1700 times less than that for CsA (Billich *et al.*, 1995) and the toxicity data also seem more favorable, with no significant change in kidney-specific parameters following 10 days of 50 mg/kg of oral NIM811 (Rosenwirth *et al.*, 1994). The same dose regimen of CsA increased serum creatinine and urea concentrations as signs of renal dysfunction. UNIL025 has a similar profile with 7000 times less immunosuppression (as compared to CsA) measured using a nuclear factor of activated T cell dependent reporter assay in Jurkat T cells (Ruegg *et al.*, unpublished observations). In addition, single dose intravenous toxicity for UNIL025 measured as LD50 in Sprague Dawley rats is between 160 and 190 mg/kg (Debiopharm, unpublished observations) which is considerably higher than that reported for CsA (104 mg/kg) (data from the SPC for Sandimmun®). The lesser degree of toxicity may facilitate administration of the analogs for neurological indications. CsA is actively transported over the cerebral capillary membranes back to the blood by the *p*-glycoprotein (P-gp), but the transport can be saturated by high concentrations of CsA and may also be blocked sufficiently by other P-gp substrates (Tsuiji

et al., 1993). Local administration in the carotid artery also saturates the transport (Yoshimoto and Siesjö, 1999) and intra-theal delivery has been successful in the treatment of transgenic SOD1 G93A mice (a model of amyotrophic lateral sclerosis) (Keep *et al.*, 2001). In animal models of traumatic brain injury, where the blood-brain barrier is disrupted due to the injury, CsA has been administered via simple intravenous injection or infusion and has displayed potent neuroprotective properties (Okonkwo *et al.*, 2003; Sullivan *et al.*, 2000). In addition, human clinical trials are underway using intravenous CsA treatment of patients with severe closed head injury (Alves *et al.*, 2003; Brophy *et al.*, 2003). If CsA proves to be clinically effective, nonimmunosuppressive cyclosporins may have a therapeutic potential in these patients by retaining the mitochondrial protection while avoiding immunosuppressive and calcineurin-related side effects.

In summary, we have shown that NIM811 and UNIL025 are potent inhibitors of mPT in brain mitochondria. This study is part of a larger project evaluating their neuroprotective potentials and we are currently exploring the neuroprotective effect of the compounds in an *in vitro* mouse hippocampal slice model of oxygen/glucose deprivation. Further *in vivo* evaluation of these interesting cyclosporin analogs will reveal if mPT is a central target for neuroprotection and if calcineurin blockade affords additional neuroprotection or counteracts the benefits by dose-dependent cytotoxicity. We envisage that nonimmunosuppressive cyclosporin analogs may be neuroprotective and may prove valuable in the treatment of severe diseases of the central nervous system.

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Using ^{31}P NMR Spectroscopy at 14.1 Tesla to Investigate PARP-1 Associated Energy Failure and Metabolic Rescue in Cerebrocortical Slices

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PARP-1 activation by H_2O_2 in an acute preparation of superfused, respiring, neonatal cerebrocortical slices was assessed from PAR-polymer formation detected with immunohistochemistry and Western blotting. ^{31}P NMR spectroscopy at 14.1 Tesla of perchloric acid slice extracts was used to assess energy failure in a 1-h H_2O_2 exposure as well as in a subsequent 4-h recovery period where the superfusate had no H_2O_2 and specifically chosen metabolic substrates. Although more data are needed to fully characterize different bioenergetic responses, a high NMR spectral resolution (PCr full-width at half-max $\approx .01$ ppm) and narrow widths for most metabolites ($< .2$ ppm) permitted accurate quantifications of spectrally resolved resonances for ADP, ATP, NAD^+/NADH , and other high energy phosphates. It appears possible to use brain slices to quantitatively study PARP-related, NAD-associated energy failure, and rescue with TCA metabolites.

KEY WORDS: Brain slice; NMR spectroscopy; poly-(ADP-ribose)-polymerase; ATP; nicotinamide adenine dinucleotide; ^{31}P .

INTRODUCTION

Studies of molecular mechanisms of hypoxic/ischemic brain injury often focus on the important role of an abundant nuclear enzyme, poly-(ADP-ribose)-polymerase-1 (PARP-1, PARP, EC 2.4.2.30). For several years in vivo studies have firmly demonstrated that in cerebral ischemia inactivation of PARP-1 reduces infarct size and improves outcome (see, for example, Eliasson *et al.*, 1997; Endres *et al.*, 1997). In healthy cells PARP-1, when activated in the nucleus by minor DNA damage, initiates repairs by first cleaving NAD^+ (β -nicotinamide adenine

dinucleotide) into its components, nicotinamide and ADP-ribose, and then attaching anywhere from a few to a few hundred ADP-ribose polymers onto histones and various proteins. It also polyribosylates itself, causing its own inactivation. Recent comprehensive reviews of PARP include those by D'Amours *et al.*, 1999; Herceg and Wang, 2001; Virag and Szabo, 2002; and Hassa and Hottiger, 2002. In healthy cells having only a small amount of DNA damage, poly-ADP-ribosylation efficiently initiates the DNA repair that occurs after enzymes in the PARG family (poly-ADP-ribose glycohydrolase) remove the poly-ADP-ribose units, undoing PARP's work. Thus poly-ADP-polyribosylation by PARP-1 lasts only for a brief time, serving as a transient activation signal to enzymes of repair.

Although there still is an incomplete understanding of the mechanisms by which PARP-1 exacerbates cell injury during and after oxygen deprivation, it is not unusual to see NAD^+ depletion listed as an important culprit. NAD^+ is a key redox participant in glycolysis and the TCA cycle, and it has been suggested that heavily damaged DNA triggers intense overconsumption of NAD^+ via

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back-to-back action of PARP-1 and PARG, i.e., by futile cycling where PARP repeatedly attaches ADP-ribose units and PARG repeatedly removes them. Additionally, NAD^+ restoration adds to energy depletion, because four ATP molecules are required to regenerate one NAD^+ (Zhang *et al.*, 1994). PARP-induced energy failure with cytosolic NAD^+ depletion—along with metabolic rescue—were recently demonstrated in neuron and astrocyte cell culture studies (Ying *et al.*, 2002), where neuron and astrocyte monolayers were treated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) or with H_2O_2 , agents known to activate PARP. Cell death in those studies was prevented or reversed by administration of PARP inhibitors or TCA cycle substrates such as pyruvate, glutamine, and α -ketoglutarate. It is important to know if this kind of PARP-associated energy failure and rescue are relevant to events occurring *in vivo*, where NAD^+/NADH reserves might be more robust. Realizing that our acute brain slice preparation provides tissue conditions closer to those *in vivo*, and that high resolution NMR spectroscopy is an outstanding tool for investigating metabolic phenomena, we sought a brain slice protocol that would exhibit PARP-associated energy failure and rescue, possibly with features recognizably similar to those seen in cell culture studies. This paper reports our findings.

MATERIALS AND METHODS

Perfused Brain Slice Preparation and Bench Top Experiments

Our protocol for obtaining, recovering, and maintaining respiring brain slices was approved by the UCSF Committee on Animal Research. Details can be found in recent publications by our group (Hirai *et al.*, 2002; Litt *et al.*, 2003). Briefly, in each experiment we used 20 cerebrocortical slices (350- μm thick) that were obtained from ten 7-day-old Sprague-Dawley rats after decapitation during isoflurane anesthesia. Slices were maintained in a 15-mL conical tube through which there was superfusion with fresh, oxygenated artificial cerebrospinal fluid (oxy-ACSF) that consisted of a modified Krebs balanced salt solution containing 124 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L KH_2PO_4 , 1.2 mmol/L MgSO_4 , 1.2 mmol/L CaCl_2 , 26 mmol/L NaHCO_3 , and 10 mmol/L glucose. The bicarbonate buffer for oxy-ACSF underwent continuous bubbling with a 95% O_2 /5% CO_2 gas mixture and maintained constant values of PCO_2 (40 mmHg), PO_2 (600–650 mmHg) and pH (7.4). The superfusion chamber was immersed in a water bath that was kept at 37°C. The oxy-ACSF flow rate was 10–15 mL/min, and slices un-

derwent 3 h of metabolic recovery before experiments began.

Experimental Design for Activating PARP and Adding Treatments

PARP activation was induced via oxidative stress from superfusion with ACSF that contained hydrogen peroxide (H_2O_2 , 2 or 4 mM). The H_2O_2 exposure was terminated after 1 h at which time a 4-h metabolic rescue was initiated. Parallel experiments were conducted with the PARP inhibitor 3-aminobenzamide (3AB, 3 or 5 mM, Sigma Chemical Co, St. Louis, MO) being present during H_2O_2 exposure. In these experiments, 3AB superfusion started 1 h prior to H_2O_2 administration and stopped when H_2O_2 exposure terminated. Immediately prior to H_2O_2 exposure the ACSF glucose concentration was lowered to 0 or 2 mM (from 10 mM). During the rescue period the ACSF contained glucose, or pyruvate (Sigma Chemical Co, St. Louis, MO), or a mixture of the two, or no energy substrate. At four time points slices were removed from the perfusion chamber, washed rapidly in 4°C normal saline, immediately frozen in liquid nitrogen, and stored at -80°C for subsequent NMR studies or Western blots: 1) $t = 0$ h, just before the 1-h H_2O_2 exposure; 2) $t = 20$ min, during H_2O_2 exposure; 3) $t = 60$ min, at the end of H_2O_2 exposure; and 4) $t = 5$ h, after 4 h of posttreatment with oxy-ACSF that contained pyruvate.

NMR Measurements—Perchloric Acid (PCA) Extraction of Slice Metabolites

Seven frozen slices from each time point were placed in a mortar containing liquid nitrogen (LN_2) and a pestle at the same temperature (-196°C). The slices were then pulverized under LN_2 , and resulting fine powder was placed in a centrifuge tube containing 5 mL of 12% perchloric acid at 4°C. After washing the tube wall with an additional 2 mL of 4°C perchloric acid, the mixture was homogenized at 4°C with an ultrasonicator (Kimble-Kontes, Vineland, NJ). After 20-min centrifugation of the mixture at 15,000 rpm at 4°C the supernatant was collected, while the pellet was resuspended with 2 mL of distilled water, ultrasonicated, and again centrifuged at 4°C. The second supernatant was added to the first, with the final mixture then being carefully, monotonically neutralized with KOH (8, 1, and 0.1 N) to the pH range of 7.0–7.4 (with no overshoots). An additional centrifugation eliminated perchlorate salts. The final supernatant was then lyophilized (BenchTop 2K Lyophilizer, Virtis, Gardiner, NY) and refrigerated at -80°C until just prior to NMR spectroscopy.

At that time each lyophilized PCA extract was dissolved in 435 μL of 99.9% D_2O . Centrifugation then followed, after which 300 μL of supernatant was taken and given a 1 mg addition of EDTA and a few additional drops of NaOD to make the final pD ≈ 7.0 –7.2. Two microliters of 330 mM methylene diphosphonate (MDP) and 1 μL of 700 mM 3-(trimethylsilyl)-tetra-deuteriosodium propionate (TSP) were added as internal NMR reference compounds having final concentrations of 2.2 and 2.33 mM, respectively. The pD was again adjusted to 7.0–7.4, if necessary, and final quantity was then loaded into a 5 mm Shigemi NMR tube (Shigemi Co, Tokyo, Japan).

NMR Measurements— ^{31}P Spectroscopy at 14.1 Tesla

NMR studies were performed in the new Mission Bay UCSF Magnetic Resonance Laboratory (<http://picasso.nmr.ucsf.edu>) using a 14.1-Tesla (600-MHz) Varian INOVA spectrometer and a customized, multi-nuclear Z-SPECT radiofrequency probe that was optimized for this project (3NG600-8, Nalorac Corporation, Martinez, CA 94553). Basic one-pulse, 90° tip angle, RF sequences were used for obtaining ^1H spectra at 599.92 MHz and ^{31}P spectra at 242.86 MHz. The extra homogeneity provided by the Shigemi NMR tubes and the Varian INOVA shimming software typically resulted in spectral linewidths of .004 ppm FWHM for ^1H water protons and .01 ppm FWHM for ^{31}P in phosphocreatine. Phosphorous spectra were obtained typically with a 1-s interpulse delay during 8-h runs.

Western Blot Analysis of Poly(ADP-ribose) Polymers as a Monitor of PARP-1

After placing three slices ($\approx 3 \times 50$ mg wet weight) from each time point in an ice-cold lysis buffer (20 mM HEPES-KOH at pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1 \times Protease inhibitor cocktail) the mixture was sonicated and then centrifuged (10,000g) at 4°C for 20 min. The resulting supernatant was used as the whole cell protein extraction. After determining protein concentrations by the Bradford method (Bio-Rad, Hercules, CA), 60 μg of protein from each sample underwent sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) on a 4–12% Bis Tris gel (Invitrogen, Carlsbad, CA). Separated proteins were transferred from the gel to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) which was then incubated for 1 h in a blocking buffer containing 5% blotting grade nonfat dry milk in 0.1 M sodium phosphate buffer (pH 7.4). Next the membrane was incubated

overnight with mouse anti-PAR monoclonal antibody 4335-MC (Trevigen, Gaithersburg, MD) diluted 1:1000, washed, and incubated for 1 h with a 1:10,000 dilution of peroxidase conjugated anti-mouse IgG (Amersham Life Science, Piscataway, NJ). Bound antibody was visualized by chemiluminescence. As a check on protein loading, β -actin, a typical “housekeeping protein,” was quantified after stripping the membrane and then following the same steps described above. PAR polymer formation was quantified by optical density (OD) measurements of intensities in the molecular weight region between 80 and 210 kDa. The OD of β -actin was measured as the internal control. Then the following ratio of ODs was determined for each time point: (PAR polymer)/(β -actin). Measured intensities for different blots on different days were normalized using quantifications of bands from a reference lane on each blot where the same amount of protein was always loaded from the same source.

RESULTS

In initial attempts to induce brain slice intracellular energy failure and NAD^+ depletion we used MNNG

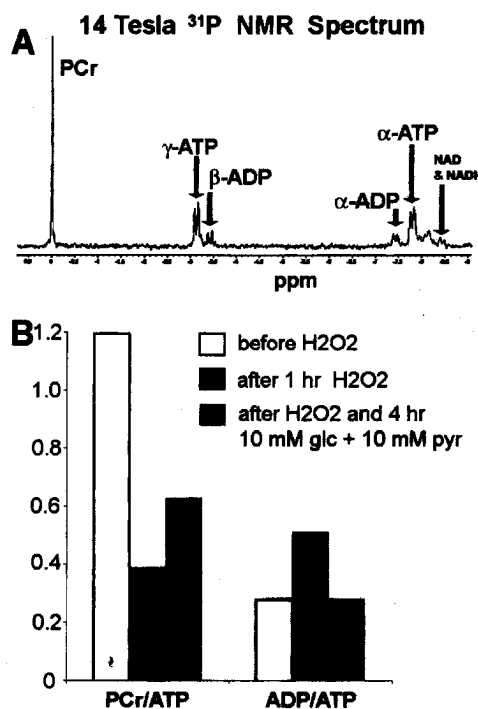


Fig. 1. (A) Region of a representative 14.1 Tesla ^{31}P NMR spectrum from slices before H_2O_2 exposure. Arrows show separately resolved ATP, ADP, and NAD doublet peaks, as described in the text. (B) Two metabolite ratios, PCr/ATP and ADP/ATP, are shown for three time points in a typical experiment, showing energy failure and recovery.

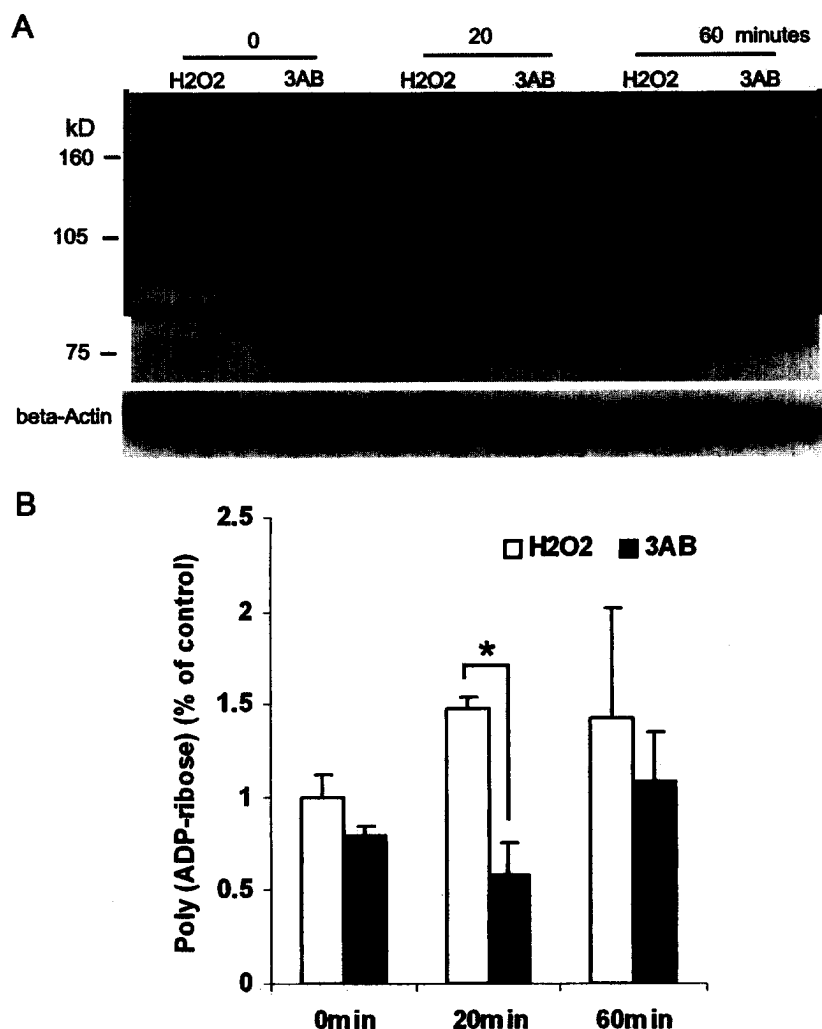


Fig. 2. (A) Representative Western blot showing PAR polymers in the molecular weight region included by the rectangle. Details are in the text. (B) Average OD ratios for three experiments, as described in the text. Bars show standard errors. * indicates significance at $p < 0.01$.

or 2-mM H₂O₂ for activation of PARP, while also maintaining our standard glucose concentration (10 mM) at all experimental times. We were successful in detecting PAR-polymer formation in those runs, but we did not start detecting energy failure and NAD depletion until the glucose concentration during H₂O₂ exposure was reduced to zero or 2 mM. Our results at this time are descriptive and qualitative, as can be seen first in Fig. 1, which shows representative data regarding energy failure, and Fig. 2, which shows representative Western blot data regarding PAR-polymer formation. The high NMR spectral resolution of Fig. 1(A) makes it easy to discern that three energy metabolites of interest, ATP, ADP, and NAD, are doublets with a frequency separation of 18.5 Hz, the value known to arise in the pyrophosphate linkages that

each contains. Each doublet is sufficiently resolved from its neighbors to permit quantification by direct integration. Figure 1(B) shows PCr/ATP and ADP/ATP ratios for three sets of slices in the same experiment, suggesting early energy failure at the 20 min point into H₂O₂ exposure and recovery afterwards. Because these ratios can be altered by the creating kinase reaction alone, metabolite quantifications rather than ratios are needed to assess energy failure. In the representative Western blot of Fig. 2(A), poly-(ADP-ribosylated)-proteins in the whole cell fraction, seen as diffuse immunoreactivity in the range of molecular weights outlined by the black rectangle, are markedly increased after 20- and 60-min exposures to 2 mM H₂O₂, but less increased, possibly unchanged, if treated as described with the PARP inhibitor 3AB (3 mM).

The same trend was found in three complete independent experiments, as shown in Fig. 2(B), where the ratio of (PAR polymers)/(β-actin) at $t = 20$ min was 1.48 ± 0.06 , compared to 0.59 ± 0.17 ($p < 0.01$) in 3 mM 3AB-treated slices; and 1.43 ± 0.59 at $t = 60$ min compared to 1.08 ± 0.27 in 3AB-treated slices.

DISCUSSION

It should not be surprising that PARP activation in robust, well perfused, well-oxygenated brain slices did not appear to trigger the type of NAD⁺ -depletion energy crisis seen in earlier cell culture studies, where availability of TCA metabolites might suffice in rescue (Ying *et al.*, 2002). Indeed, PARP-1's actions are intertwined with many nonmetabolic mechanisms known to be important in hypoxic/ischemic injury, these including apoptosis and activation of rescue kinases and inflammatory mediators (Chiarugi and Moskowitz, 2003; Narasimhan *et al.*, 2003; Yu *et al.*, 2003). However, the brain slice system did reveal that it is amenable to accurate quantification of metabolite changes, and that with fine tuning of the model, energy failure and NAD⁺ depletion (data not shown) can be made to occur. We note also that NMR spectroscopy, unlike enzymatic quantifications of NAD⁺, measures only that NAD⁺ which is freely mobile. Thus it seems possible that interesting information might come from comparisons of NMR and non-NMR NAD⁺ quantifications. We find it noteworthy that total NAD⁺ quantifications, without mention of redox ratios, have been emphasized in recent assessments of bioenergetic viability. It has long been known that there are many intricacies by which cells maintain a cytosolic [NAD⁺]/[NADH] ratio appropriate for supplying lots of NAD⁺ to glycolysis, while also maintaining a different mitochondrial ratio appropriate for supplying NADH to the electron transport chain (Newsholme, 1973). The use of ¹³C-labeled nutrients in NMR bioenergetic studies might help provide insight to such things, because new high resolution NMR methods permit ¹³C-

labeled glycolytic and TCA intermediates to be identified and monitored via ¹H detection, which has considerably higher sensitivity (Burgess *et al.*, 2001). In any event we find the preliminary data of this paper to be very encouraging about the establishment of brain slices paradigms for mechanistic studies, because PARP activation rapidly impairs glycolysis if exogenous TCA substrates are not available.

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Development and Initial Characterization of Xenomitochondrial Mice

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Xenomitochondrial mice harboring trans-species mitochondria on a *Mus musculus domesticus* (MD) nuclear background were produced. We created xenomitochondrial ES cell cybrids by fusing *Mus spretus* (MS), *Mus caroli* (MC), *Mus dunni* (Mdu), or *Mus pahari* (MP) mitochondrial donor cytoplasts and rhodamine 6-G treated CC9.3.1 or PC4 ES cells. The selected donor backgrounds reflected increasing evolutionary divergence from MD mice and the resultant mitochondrial–nuclear mismatch targeted a graded respiratory chain defect. Homoplasmic (MS, MC, Mdu, and MP) and heteroplasmic (MC) cell lines were injected into MD ova, and liveborn chimeric mice were obtained (MS/MD 18 of 87, MC/MD 6 of 46, Mdu/MD 31 of 140, and MP/MD 1 of 9 founder chimeras, respectively). Seven MS/MD, 1 MC/MD, and 11 Mdu/MD chimeric founder females were mated with wild-type MD males, and 18 of 19 (95%) were fertile. Of fertile females, only one chimeric MS/MD (1% coat color chimerism) and four chimeric Mdu/MD females (80–90% coat color chimerism) produced homoplasmic offspring with low efficiency (7 of 135; 5%). Four male and three female offspring were homoplasmic for the introduced mitochondrial backgrounds. Three male and one female offspring proved viable. Generation of mouse lines using additional female ES cell lineages is underway. We hypothesize that these mice, when crossbred with neurodegenerative-disease mouse models, will show accelerated age-related neuronal loss, because of their suboptimal capacity for oxidative phosphorylation and putatively increased oxidative stress.

KEY WORDS: mtDNA; cybrid; xenomitochondrial; ES cells; mouse models; neurodegeneration.

INTRODUCTION

Mitochondrial DNA (mtDNA) diseases in humans are pleiotropic, with neurological pathology common. All mtDNA gene products comprise subunits of the multi-

meric oxidative phosphorylation (OXPHOS) complexes. The reasons some mtDNA mutations affect certain neuronal populations, while others lead to muscle or endocrine disease, for example, remain mysterious (see DiMauro and Schon, 2001). The potential contribution of OXPHOS defects to the expression of common sporadic neurodegenerative diseases is also often discussed (see Manfredi and Beal, 2000), yet there is no mouse model with mtDNA-based OXPHOS impairment available to directly test this hypothesis.

We have recently demonstrated the feasibility of replacing the endogenous mtDNA of the laboratory mouse, *Mus musculus domesticus*, with that of different mouse species of increasing evolutionary divergence (McKenzie *et al.*, 2004). Our objective in creating such xenomitochondrial mice is to enable a model system where decremental impairments of mtDNA-based

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OXPHOS exist in viable mice. Such mice will then allow direct investigation of different degrees of OXPHOS impairment in tissue pathogenesis, and furthermore allow superimposition of such impairment on nuclear gene models of neurodegenerative disease in crossbreeding experiments.

Since female embryonic stem (ES) cells have not been widely developed for mouse transgenesis, we have been limited by the relatively poor germline success rate of existing lines, having obtained only a few live male animals to date. We have now succeeded in obtaining a homoplasmic female founder, bringing us an important step closer to the creation of mouse lines. Here we describe the genetic basis for the models and their potential utility for studying subtle to mild metabolic disturbances based on mtDNA defects, and for exploring hypothetical OXPHOS contributions to pathogenesis in neurodegenerative diseases.

MATERIALS AND METHODS

Cell Lines

Primary fibroblast lines were created from a 2-day-old laboratory mouse (*Mus musculus domesticus*, CBA \times C57BL/6 F1 cross), a 5-week-old *Mus spretus* mouse (*SPRET/Ei*, a gift from Dr. Simon Foote, WEHI, Melbourne, Australia), and newborn pups from *Mus pahari* (*Mus pahari/EiJ*) and *Mus caroli* (*Mus caroli/EiJ*) bred from animals purchased from the Jackson Laboratory (McKenzie *et al.*, 2003). A *Mus dunni* primary fibroblast cell line, designated III8C, was obtained from the American Type Culture Collection (Manassas, VA). The mouse ρ^0 cell clone LMEB3 was derived from the parental line LMTK⁻ by exposure to ethidium bromide (Trounce *et al.*, 2000; see also Pinkert and Trounce, 2002).

Production of Xenomitochondrial L-Cell Cybrids

Mouse cybrids were produced by enucleation of mitochondrial donor cells and fusion of the cytoplasts with mouse ρ^0 cells followed by selection for respiratory competent transformants. Procedures were described in detail previously (McKenzie and Trounce, 2000). Cells used as mitochondrial donors included the MD, MS, Mdu, MC, and MP primary fibroblast lines described above.

Production of Xenomitochondrial ES Cell Cybrids

The mouse female ES cell line CC9.3.1 was derived from a 129SvEv-Gpil^c (129S6-Gpil^c) embryo

(A. Bradley, Baylor College of Medicine, Houston, TX) and was previously shown to have the potential to contribute to the female germ line and produce normal fertile females (Sligh *et al.*, 2000). ES cells were treated with rhodamine 6-G for 3 days prior to fusion with cytoplasts made by enucleation of L-cell xenocybrids, and cybrids selected as previously described (McKenzie *et al.*, 2004).

mtDNA Genotyping by Restriction Enzyme Analysis and DNA Sequencing

A 461-bp mtDNA fragment containing part of the D-loop and the tRNA-Phe gene was amplified as previously described (McKenzie *et al.*, 2003), and restricted with either StyI or Bsu36I to generate species-specific restriction fragments. The complete cytochrome *b* gene was also amplified from each species' DNA and sequenced (McKenzie *et al.*, 2003).

Production of Transmitochondrial Mice

Procedures used for the collection and manipulation of blastocysts have been described in detail elsewhere (Polites and Pinkert, 2002). Recently, xenomitochondrial ES cell injections into mouse blastocysts were also described (Pinkert and Trounce, 2002, in press; McKenzie *et al.*, 2004). Briefly, progeny resulting from ES cell injections were initially identified by coat color chimerism (chimeras exhibited varying percentages of agouti fur) and all founders were genotyped using species-specific PCR analyses. Tail DNA from non-coat-color chimeras (black coat color) were also analyzed by PCR for chimerism; Clone S2 male #22 was identified as carrying *Mus spretus* mtDNA in this fashion. Dead or euthanatized animals were dissected and the DNA was extracted from various tissues as described above. PCR and restriction digests were performed to confirm mitochondrial genotypes and tissue distribution.

Founder chimeric (heteroplasmic for MS or Mdu mitochondria) females (G₀) were mated to control C57BL/6NTac males (homoplasmic for MD mitochondria). Offspring (G₁) were homoplasmic as expected, either for the host blastocyst or ES-cell-derived mitochondria. Tail biopsies were taken and DNA extraction, PCR, and restriction digests were performed for confirmation of genotype.

All mice were maintained in a specific pathogen-free barrier facility and were anesthetized by Avertin (2,2,2-tribromoethanol) injection during any potentially painful procedure. Mice were monitored carefully after

Table I. Amino Acid Differences in Species-Specific mtDNA Translation Products (Extrapolated from Cytochrome *b* Sequences)

mt gene product	# Amino acids	Number of amino acid replacements compared with <i>Mus musculus</i>				
		<i>M. spretus</i>	<i>M. dunni</i>	<i>M. caroli</i>	<i>M. pahari</i>	<i>Rattus</i>
nad1	315	5	9	10	15	32
nad2	345	14	24	27	41	88
coxI	514	2	4	4	7	14
coxII	227	1	1	1	1	3
atp8	67	2	4	4	7	14
atp6	226	2	3	4	6	12
coxIII	261	1	2	3	4	9
nad3	114	2	4	5	7	15
nad4L	97	2	4	4	7	14
nad4	459	9	16	18	27	59
nad5	607	21	36	41	61	133
nad6	172	5	9	11	16	34
cytb	381	4	7	8	12	26
Total	3785	70	123	140	211	453

Note. Values for cytochrome *b*, shown in bold, are from complete sequences. Other values are extrapolations based on the relative divergence from *Rattus norvegicus* (*Rattus*).

anesthesia until full recovery. Mice were euthanatized by CO₂ inhalation or Avertin sedation and cervical dislocation. All procedures followed the AVMA guide per institutional guidelines.

RESULTS

We first produced mouse L-cell cybrids harboring mitochondria from the four species of interest. The species were chosen based on increasing evolutionary divergence so that increasing mismatching of the foreign mtDNA subunits and the *Mus musculus* nuclear-encoded subunits would result in mild, increasing OXPHOS defects (McKenzie *et al.*, 2003). These L-cells are amenable for large-scale cultures needed to isolate sufficient intact mitochondria for full OXPHOS characterization. As discussed in detail in McKenzie *et al.*, (2003), these xenocybrids indeed showed a graded mild respiratory deficiency. Only the MP cybrids showed a significant defect of an OXPHOS enzyme, with a 40% deficiency of cytochrome oxidase activity amid preserved activity of other complexes. Yet, all four cybrids showed elevated lactate production around 2-fold in MS and MC cybrids and 2.5-fold in Mdu and MP cybrids (McKenzie *et al.*, 2003). This indicated that subtle OXPHOS defects existed in these cybrids. Increasing the mismatch level by using further diverged mtDNA donors (e.g., *Rattus norvegicus*) resulted in severe OXPHOS defects (McKenzie *et al.*, 2003). The approximate num-

ber of amino acid substitutions in mtDNA genes of the four mouse species used, based on the cytochrome *b* sequences and compared with *Mus musculus*, are shown in Table I.

We then produced ES cell cybrids with all four constructs, by first treating the female ES cell line CC9.3.1 with rhodamine 6-G prior to fusion with cytoplasts from the mitochondrial donor cybrids. These cybrids were genotyped to identify homoplasmic or heteroplasmic clones, and chosen clones injected into mouse blastocysts.

Eleven xenomitochondrial ES cell clones (nine homoplasmic and two heteroplasmic clones) were injected into mouse blastocysts (see Table II). In total, 1684 blastocysts were injected yielding 319 pups. Based on coat-color chimerism and/or confirmatory PCR genotyping, 56 chimeras were obtained, with percentage of chimerism ranging from approximately 1% to over 99%. Nineteen chimeric females were weaned and bred to control C57BL/6NTac males (Table III). Of these breeders, 18 pairs were fertile, producing 583 pups. Five of the female chimeras (all resulting from injection of homoplasmic xenomitochondrial ES cells) that were 1–90% chimeric on the basis of coat color proved germline competent; however, the yield of homoplasmic mutants was low. Of their 135 combined/analyzed offspring, there were 4 males and 3 females produced that were homoplasmic for the introduced mitochondrial genotype. While most germline-competent chimeras delivered a single homoplasmic mutant, one female did deliver 3 *M. dunni* xenomitochondrial offspring; however, all three were dead at birth. At this

Table II. Generation of Chimeric Heteroplasmic Founder Mice

Clone	Ova injected	Founders	Weaned	Chimeras
S2 (<i>Mus spretus</i>)	227	66	42	13/61
S3 (<i>Mus spretus</i>)	194	28	14	5/26
C8 (<i>Mus caroli</i>)	23	4	4	1/4
S15 (<i>Mus caroli</i>)	154	30	11	5/26
C23 (<i>Mus caroli</i>)	152	22	7	0/16
D6 (<i>Mus dunni</i>)	169	43	30	7/38
D7 (<i>Mus dunni</i>)	589	116	59	24/102
P5 (<i>Mus pahari</i>)	63	5	0	1/5
P12 (<i>Mus pahari</i>)	45	0	0	0
P17 (<i>Mus pahari</i>)	59	5	1	0/4
P24 (<i>Mus pahari</i>)	11	0	0	0

Note. Chimeric heteroplasmic founder mice were generated from different ES xenomitochondrial cybrid clones. *Mus spretus* and *Mus dunni* data were previously reported (McKenzie *et al.*, 2004). Using restriction digest analysis, injected clones were homoplasmic for the respective species mtDNA with the exception of two *Mus caroli* cell lines (C15 and C23) that were purposefully heteroplasmic (approx. 50% *Mus caroli* and 50% *Mus musculus domesticus* mitochondria) toward development of mitochondrial dynamics/competition model systems.

Table III. Breeding of Chimeric Founder Females

Clone	Female (% chimerism)	Offspring	Germline transmission	Germline offspring, status
S2 (<i>Mus spretus</i> homoplasmic clone; 13 chimeras in total)				
	13 ♀ (20)	19	0/19	
	26 ♀ (15)	54	0/47	
	33 ♀ (5)	10	0/5	
	37 ♀ (1)	20	1/15	1 ♂, viable
S3 (<i>Mus spretus</i> homoplasmic clone; 5 chimeras in total)				
	02 ♀ (10)	46	0/35	
	16 ♀ (20)	29	0/27	
	17 ♀ (5)	9	0/9	
C15 (<i>Mus caroli</i> heteroplasmic clone; 5 chimeras in total)				
	06 ♀ (40)	32	0/17	
D6 (<i>Mus dunni</i> homoplasmic clone; 7 chimeras in total)				
	01 ♀ (20)	48	0/48	
	27 ♀ (20)	33	0/33	
D7 (<i>Mus dunni</i> homoplasmic clone; 24 chimeras in total)				
	01 ♀ (90)	31	0/26	
	02 ♀ (10)	32	0/29	
	34 ♀ (80)	34	1/33	1 ♂, viable
	35 ♀ (80)	No offspring		
	55 ♀ (90)	31	3/23	1 ♂ + 2 ♀ dead
	62 ♀ (95)	68	0/38	
	63 ♀ (85)	64	1/51	1 ♂, viable
	87 ♀ (85)	42	0/33	
	89 ♀ (80)	21	1/13	1 ♀, viable

Note. Weaned chimeric founder females (heteroplasmic for Ms, Mc, or Mdu mitochondria) were bred with C57BL/6NTac (B6) males to generate heteroplasmic or homoplasmic offspring. Viable xenomitochondrial offspring were all derived from homoplasmic cell lines and exhibited agouti fur pigmentation. Homoplasmy was confirmed by PCR and Southern blot analysis. All other offspring from female lineages bred to control B6 males had black fur. Chimera S3 #16 died at 5 weeks of age. Ovaries were harvested and transferred to nude female recipients to attempt lineage rescue. Pups from chimera D7 #55 died or were killed within 24 h of birth; gender and homoplasmy were confirmed by PCR and/or Southern blot analysis. Portions of these data were previously reported (McKenzie *et al.*, 2004).

time, *M. dunni* female #89 and her homoplasmic daughter represent the first mutant lineage that has the capacity to go beyond the first generation.

It should be noted that male chimeras and male first-generation homoplasmic mutants were all bred to control C57BL/6NTac females. Some of the chimeric males were infertile; others sired only black offspring—none of which harbored introduced mitochondria. In contrast, the homoplasmic males were fertile, and while both black and agouti offspring were obtained, as expected, none harbored the introduced (*M. spretus* or *M. dunni*) mitochondria, on the basis of PCR analyses.

While survival of pups to weaning was lower than anticipated, survival rates of control litters on unrelated projects were correspondingly low and likely reflected transition into a new animal facility. Over the last year, survival rates across all projects have increased and now reflect a seasonally correlated pattern (but under a 15% loss across the last year).

It appears that chimeric founders and homoplasmic male offspring have similar lifespans when compared to other mice within the same vivarium suite (but unrelated to this project). The one observation that has not been fully explained or characterized relates to three chimeric females (two germline and one nongermline) that were moribund or found dead following the birth of their third or fourth litters. While necropsy and histopathological reports point to a periparturient dystocia as a likely cause of death (C. A. Pinkert, R. B. Baggs, and I. A. Trounce, unpublished data), it is unclear if this phenomenon is directly related to their mitochondrial genetics and a stress susceptibility triggered by the periparturient cascade of obviously energy-dependent events.

DISCUSSION

Despite much interest in the potential role of mtDNA variants as contributors to neurodegenerative disease, there remains no technology for creating targeted mutations in the mtDNA. The number of mouse cell mtDNA mutants described in the literature is also quite limited, and of those characterized for OXPHOS enzymology most appear to result in severe defects (see Trounce and Pinkert, in press). Faced with these barriers, we undertook the approach of introducing foreign mouse species mtDNAs directly into zygotes and ES cells. Initially, methods for mitochondrial isolation and inter-species transfer were devised using mitochondria injection into mouse zygotes (Pinkert *et al.*, 1997). Transmitochondrial founders were obtained and germline transmission of the heteroplasmic state was observed in maternal lineages (Irwin *et al.*, 1999;

Pinkert and Trounce, 2002). To pursue homoplasmic models, we then used the cybrid route, creating cybrid ES cells followed by blastocyst injection, chimera production, and breeding to identify homoplasmic transmitochondrial offspring (McKenzie *et al.*, 2004).

The CAP^R mutant used by Sligh *et al.* (2000)—to produce the first transmitochondrial homoplasmic mice—had moderate OXPHOS defects consequent to the translation defect associated with the mtDNA 16S rRNA mutation (Levy *et al.*, 1999). Yet, the homoplasmic pups produced by Sligh *et al.* (2000), died shortly after birth with a metabolic acidosis and cardiomyopathy. In view of these important findings, we have aimed for the generation of viable mouse lines with mild OXPHOS defects. Our most divergent *Mus musculus* xenocybrid, harboring *Mus pahari* mtDNA, appears to result in a less severe defect compared to the multiple partial OXPHOS complex defects seen in the CAP^R mutant. Our other xenocybrids should show only very mild defects. How then may these be relevant or informative in modelling mtDNA and OXPHOS contributions to neurodegenerative diseases?

The link between inhibition of OXPHOS complex I and Parkinson's disease (PD) is now firmly established by important toxin models of this disease (see Dawson and Dawson, 2003), first using MPTP in mice (Heikkilä *et al.*, 1984) and later rotenone in rats (Betarbet *et al.*, 2000). An emerging view is that the increase in reactive oxygen species (ROS) consequent to the complex I inhibition is more important to dopaminergic cell loss in these models than the decreased ATP production per se (see Sherer *et al.*, 2003). A link between mtDNA polymorphisms in humans and increased or decreased risk of PD can therefore be hypothesized, and our models with increased polymorphisms in the mtDNA-encoded complex I genes may be useful in further probing such links. Claims have indeed been made for association of mtDNA variants with both sporadic PD and Alzheimer's disease (Trimmer *et al.*, 2004a,b), but to date no specific variants have been identified.

The xenomitochondrial approach introduces an "uncontrolled" range of amino acid variation, in the sense that we do not choose the changes. Table I shows the approximate degree of this variation expected for the four species used in this work, compared to *Mus musculus*. Sequencing of the complete mtDNAs of the species used is underway (W. K. Pogozelski, C. A. Cassar, I. A. Trounce, and C. A. Pinkert, unpublished data), so the table represents extrapolation from the limited comparative sequence data we have to date. The most divergent construct, using *mus pahari* mtDNA, has around 1 in 20 amino acids substituted. The most conserved construct, with *Mus spretus* mtDNA, has around 1 in 50 amino acids substituted. It

can be appreciated from the table that these replacements are not distributed evenly throughout the mtDNA, with the complex I genes ND5, ND2, and ND4 accounting for more than half, while genes such as COI, COII, COIII, and ATP6 show a much lower level of replacements. The *Mus pahari* cybrids show a mild cytochrome oxidase deficiency with apparently normal function of the other respiratory chain complexes, while the other three constructs show OXPHOS enzyme function that is not significantly different to control cybrids in vitro (McKenzie *et al.*, 2003). Using cell lactate production as a more sensitive indirect measure of oxidative ATP production, all constructs showed significantly elevated lactate compared with the wild-type cybrid (McKenzie *et al.*, 2003).

These initial findings suggest that polymorphism in the complex I genes is better tolerated than that in the more highly conserved complex IV and ATP synthase genes. Our homoplasmic MS male mouse (70 amino acid replacements) and two Mdu males (120 replacements), have shown outwardly normal development and aging in our studies to date. We anticipate that the MC and MP mice will begin to exhibit signs of age-related disease, but full evaluation of these different models awaits the production of females and mouse lines.

Production of transmitochondrial mouse models has proven to be a daunting challenge. In addition to early microinjection studies, and the ES-cell-based work, we have also attempted nuclear transfer studies to try to expedite development of xenomitochondrial models (K. Takeda *et al.*, unpublished data).

Breeding of founder females allowed production of germline mice, albeit at low rates, most likely related to the low germline competency of the ES cells. Alternatively, it is possible that homoplasmic offspring may have to surmount a metabolic crisis based on a mitochondrial-nuclear mismatch that is developmentally critical as noted in children. Boles and coworkers (2003) have described a severe but reversible cardiomyopathy in a number of infants, that was associated with an mtDNA D-loop heteroplasmy. Reversible cytochrome oxidase deficiencies, in particular, have also been previously described (see DiMauro and Schon, 2001). As such, it is possible that a comparable situation exists in our mice. If this hypothesis holds, then the homoplasmic first-generation offspring represent those animals that survive beyond the early postnatal period surmounting a prenatal or perinatal metabolic crisis. Following this time point, they then develop on through adulthood, where both *Mus spretus* and *Mus dunni* "xenomitomice" appear quite normal.

It is most exciting that a live female has been produced indicating that if an animal must surmount a putative metabolic crisis as we have hypothesized, then

females may very likely have a comparable viability to the males that we preliminarily characterized. While this female may represent a developmental anomaly, she does illustrate that the model is valid. Further studies with new murine ES cell lineages may also heighten our overall experimental efficiencies (C. A. Cassar, E. M. Vollmers, I. A. Trounce, and C. A. Pinkert, unpublished data). In addition, from preliminary analysis of chimeric founders, it appears that the animals on hand have preferentially maintained wild-type mitochondrial genotypes at the expense of the introduced (mutant) mitochondrial genomes. This observation would also account for the limited germline transmission results obtained to date.

Because of the unique features of mitochondrial genetics, nuclear gene knockout mice will not supersede such models, and the creation of mitochondrial mutants will shed additional light upon mitochondrial function and dynamics of heteroplasmy. Such mice will also be of great interest to researchers of Mendelian and sporadic neurodegenerative diseases where oxidative stress or secondary mitochondrial impairment is implicated. Lastly, by cross-breeding mutant models with nuclear gene knockout mice, a new realm of experimental genetics will be available for investigating nuclear-mitochondrial interactions.

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